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International Symposium on Rice Research in the Era of Global Warming

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Preface

Climate change due to global warming may be one of the greatest threats facing the planet, as we have witnessed increasing temperatures in various regions and increasing extremities of natural disasters in recent years. The potential impacts of global warming on agriculture are in many ways, including crop production, agricultural practices, environmental effects, and ecosystems. This symposium was held on October 6-7, 2009 to discuss effects of climate change on rice production and identify research needs to deal with the inevitable threats of global warming.

Growing rice crop is subject to numerous stresses such as flooding, drought, chilling injury, heat injury, salt injury and pest problems, including weeds, insects and pathogens. Efforts to overcome these stresses in the past have been through development of stress-tolerant varieties by conventional breeding and improvement of cultural techniques. Application of modern technologies such as exploitation of DNA markers in rice genome for rice breeding will likely accelerate the development of new rice varieties that are tolerant to environmental stresses.

Since rice is the most important food crop in Taiwan, our research efforts on this crop have been intensive in the past, especially in the fields of breeding, pest management (weeds, insects and diseases) and cultural practices. Several rice varieties with high yield and superior quality have been developed through conventional breeding by researchers at the Taiwan Agricultural Research Institute (TARI) and other Agricultural Improvement Stations. During the past ten years, the rice breeding program at TARI has expanded further to incorporate new molecular techniques for improvement of rice production. Numerous researchers in other rice-producing countries are also actively pursuing studies on the use of DNA technologies for rice breeding. At this 2-day symposium, we are fortunate to invite ten speakers, who are renowned scientists in the field of rice research, from Taiwan,

Philippines, and Japan to discuss issues related to rice production under harsh climate. Their presentations published in the proceedings would benefit not only all the participants but also many others involved in the research of rice crop.

As the Director General of TARI, I would like to express my gratitude to the speakers for their contributions and to members of TARI for organizing this symposium and editing the proceedings. Financial supports for the symposium and the proceedings are provided by the Council of Agriculture, Taiwan.

Dah-Jiang Liu, Ph.D.

Director General
Taiwan Agricultural Research Institute
Council of Agriculture

Opening Ceremony

Welcome Address

Dr. Dah-Jiang Liu
Director General
Taiwan Agriculture Research Institute
Council of Agriculture

Dr. Wu, Research Fellow of the Academia Sinica and a very respected rice scientist; Dr. Chang, Director of the Department of International Affairs of the Council of Agriculture; Dear Participants; Honorable Guests; Ladies and Gentlemen. Good morning.

Welcome to Taiwan Agricultural Research Institute for the International Symposium on Rice Research in the Era of Global Warming. We are here for the opening ceremony of the Symposium, and you know that typhoon Parma has been lingering in the Bashi Channel between the Philippines and Taiwan for several days. The weakening typhoon persists. It is still there. There is an old Chinese saying: "Old friends come to visit me in the midst of a thunderstorm", implying the precious friendship among old pals and the happiest mood of the master. Here at TARI, as the host of the Symposium, with all my honor and pleasure, I sincerely welcome all of you coming to this Institute for the very important Symposium.

We are all familiar with the meaning and consequences of global warming in the long run. Scientists around the world, including those in Taiwan, are working hard to get clear the responses of crops to the environmental changes. Genetic variation in tolerating rather extreme climatic or soil conditions has been evaluated. In the past ten years, we have built up the mutation pool with over 3,000 lines of the

japonica rice Tainung 67 in order to create more genetic variability. Together with Dr. Johnson Wang of the National Chung-Hsing University, and in cooperation with the International Rice Research Institute, we have also established the IR-64 mutation pool with more than 600 lines at the F5 or F6 generation; and more lines are coming. Part of the mutated lines has been screened for their performance under unfavorable environmental conditions; and breeding programs with these materials are being carried out.

During the past 25 years in Taiwan, the most important goal of rice breeding has been the development of varieties of high eating quality. This Institute, along with our Chiayi branch station and the seven independent district agricultural improvement stations, that is, a total of 9 institutions, have been working competitively on the breeding of high quality rice. For an island as small as Taiwan, it might not be sensible to invest so much money and manpower into this single breeding program. Taiwan Agricultural Research Institute, at the headquarters here, has decided that, starting from next year, we will no longer make any new cross for the development of high quality rice. Rather, we will shift our resources to the breeding of rice varieties adaptive to high temperature or high salinity, or tolerant to drought or low water supply condition. More efforts will also be put on the utilization of molecular markers to facilitate the process of our rice breeding programs.

Taiwan has a long history of cooperation with Japan, the Philippines and the United States on researches of not only rice, but also other subjects of agricultural production. I sincerely wish that by holding this symposium, more meaningful collaboration on issues of global warming can be developed. By working together,

we should be in a much better position to meet the challenges of the changing environments.

I thank the Council of Agriculture for supporting rice research, international cooperation, and this Symposium; and to Dr. Chang for her presence at the opening ceremony. I would like to appreciate Dr. Wu, Director of the Biotech Division of TARI, and his staffs for making all the preparations and arrangements. I fully understand and appraise their hardworking during the past several months.
I would also like to thank specially to Dr. Wu Hong-Pan of Academia Sinica. He has done so much to help the materialization of this Symposium in such a short period of time, not to mention Dr. Wu's long-time and critical contribution to rice research in Taiwan.

Of course, I thank you, all the speakers and participants in this room. You will certainly make great contributions and benefit from each other in our future rice studies aimed to cope with the impacts of global warming.

Please enjoy your stay in this Institute for the next two days. To our foreign friends, have a wonderful time in this country. I assure you that typhoon Parma will be gone by tomorrow so that your trip back home will certainly be a very safe one.

Thank you very much.

Opening Remarks

Dr. Su-Sang Chang
Dircetor General
International Affairs Department
Council of Agriculture

Dr. Hong-Pang Wu, distinguished Research Fellow of Academia Sinica, Dr. Liu, Director General of Taiwan Agricultural Research Institute, all honorable guests and participants, Ladies and Gentlemen, Good Morning!

On behalf of the Council of Agriculture of Taiwan, I am very glad to be here, and it's a great honor for me to address you as part of the opening ceremony for the International Symposium on Rice Research in the Era of Global Warming. I would also like to extend my warmest welcome to all of you, especially those friends coming a long way to this country from Japan and the Philippines.

Global warming has been an issue of worldwide concern for a long time. Its impact on the global ecology and human beings is not as far away as we thought before. Actually, we are facing the global warming right now and it has already been threatening our day-to-day living to an extent beyond what we could have imagined a few years ago. Earlier this year in July, we worried about the possible drought which could imperil the transplanting of the second crop rice in Taiwan. Then, a few days later in August, the struck of the severest flood we have ever encountered in our history took hundreds of lives away and wiped out thousands of hectares of farmland and fish ponds in just two days. This example, along with so many other climatic disasters occurred in many countries in recent years, forced us to face

seriously the inevitable consequences of atmospheric change or global warming to be happened in the near future.

The production of agricultural crops depends on the adequacy of environmental conditions. As a result of evolution, almost all crops can grow normally only in a rather narrow range of temperature. With the trend of increasing temperature and decreasing supply of available irrigation water due to global warming, agricultural harvest and food supply, as well as human life and welfare as a whole, is an urgent issue must be taken into consideration by agricultural scientists in the whole world. No country can consider herself an exception.

For the climate changing dramatically and unpredictably, our government has assigned us missions to develop various crop varieties of stress-tolerance to the environment. Because the earth warming is a global problem, it may results in frequent occurrence of alternant heat waves, droughts, floods, plant diseases, and the winter may be shorter but colder, and so on. The resulting problems are complex and cross-interacting each other. As agronomists and plant physiologists and pathologists, in the scene, we have been capable of contributing our efforts on rice breeding and cultivation. Thus an important purpose of this symposium is to gather the experts of this field that we will be acquainted with each other through the talks and discussion activities. I deeply hope after this symposium we may work together in the future, because we have the same goal to overcome the inevitable obstacles.

I would like to thank the Symposium Committee for organizing this fantastic symposium, and especially thanks to all outstanding scientists and experts from abroad and Taiwan for attending this symposium.

In this room, I see many agricultural experts from different regions of Taiwan, some of you I am very familiar with, because we have worked together for a long time for Taiwan’s agriculture. We have just heard Dr. Liu’s address reviewing the history of

rice research in Taiwan. This is just one of the remarkable achievements of our agricultural research in Taiwan. I am truly thankful to all of you for contribution to new scientific knowledge and increase our farmers' income. Today, I am also very happy to see so many young friends in this room. You are the new generation and new blood for our efforts to improve sustainability of agriculture in Taiwan.

Particularly, I would like to thank all the invited speakers for your contributions to this symposium. I wish all of you will enjoy the sessions of this symposium and establish friendships and future collaborations after this symposium.

Presentation Papers

International Symposium (2009)
Rice Research in the Era of Global Warming
1~9

Effect of global warming on rice culture and adaptive strategies

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ABSTRACT

The Fourth Assessment Report of Intergovernmental Panel on Climate Change in 2007 implied the increasing trend in global temperatures associated with anthropogenic greenhouse gases concentrations. In recent years, negative effect by high temperature to rice yield and grain appearance quality is recognized even in sub-tropical and temperate areas. High temperature induced sterility is most serious threat by high temperature on yield reduction. Occurrence of chalky grain is now crucial issue in Japan. Although high temperature during early ripening stages is a major trigger for chalky grain, other climatic conditions such as radiation, agronomic techniques and rice varieties are also responsible. This indicates the importance of integrated measure of variety and crop management to mitigate negative effect of high temperature.

Key words: rice, high temperature, grain quality

INTRODUCTION

The Fourth Assessment Report of Intergovernmental Panel on Climate Change (IPCC) in 2007 strongly implied that the increasing trend in global temperatures since the mid-20th century is the result of increase in anthropogenic greenhouse gases concentrations. High atmospheric CO_2 leads to the increase in biomass

production and yield through enhanced photosynthesis. On the other hand, it has been known that high temperature above optimum has negative effect on yield and grain quality in rice by inducing decline of grain setting, reduced grain weight, increased respiration, and also through aggravating biotic stress. Importantly, high temperature indirectly suppresses rice production by increasing water demand in water-limited area. Coping with these constraints derived from rising temperature will maximize the beneficial effect by CO_2 increases. Developing adaptive strategies to increasing temperature by understanding the response of rice to high temperature are crucial challenges in rice research. This paper reports on current knowledge on physiological response of rice to high temperature mainly based on experiences in Japan and discuss adaptive strategy.

YIELD AND GRAIN SETTING

Spikelet formation

The effect of temperature on yield appears through the changes in biomass production and harvest index. Generally, harvest index is more fluctuant as compared with biomass production due to higher sensitivity to temperature in spikelet formation, grain setting and grain filling. The study in western Japan (Suzuki 1980) showed that high temperature has positive effect on biomass production at early stages by enhancing tillering, leaf expansion and nutrient uptake. While, positive effect by higher temperature on biomass is less or becomes negative in later stages due to larger burden from increased maintenance respiration associated with excessive growth, faster senescence, and shortened growth period.

The factors causing reduction in harvest index by high temperature include decreased number of spikelet, sterility, and reduced single grain weight. Lowered spikelet formation efficiency, which is defined as spikelet number per unit biomass or unit N uptake at heading stage, is partly attributed to increased

proportion of unproductive tiller, lowered number of spikelet per panicle caused by excessive vegetative growth and reduced N uptake. Yield decline trend with decreasing biomass and spikelet number was observed in long-term experiment in the Philippines (Peng et al 2001). It appeared to be associated with increase in daily minimum temperature. Physiological mechanisms underlying specific effect of high night temperature on the spikelet formation remain unclear for further study.

High-temperature-induced sterility

Floret sterility induced by high temperature above 35 ^{o}C (Satake and Yoshida 1978) is most serious threat to reduce grain yield. High temperature-induced sterility has been reported in the tropics, and recently in China (Wang 2002). In the hot summer of 2007, increase in sterility rate was observed in farmers fields in central Japan (Hasegawa et al 2009). The most sensitive stage to high temperature-induced sterility is anthesis followed by young microspore stages (Satake and Yoshida 1978). High day temperature at anthesis time induces sterility by reducing the number of pollen grain shed on stigma. Morphologies of anther which affect theca dehiscence are proposed to be responsible for genotypic difference in sensitivity of pollen shedding, such as thickness of locule wall, length of dehiscence at basal part of theca (Matsui et al 2001, 2005). It was also proposed that the traits that flower early morning or night, which is found in cultivated rice species and wild relatives, may also be useful to escape from high day temperature (Nishiyama and Blanco 1980).

We need more information on critical temperature and stages under field conditions to assess the risks of sterility under global warming. Field observation in open field (Hasegawa et al 2009) implied that sterility rate with air temperature above 35 ^{o}C was considerably lower than that predicted from the results obtained in chamber experiment. Possible factor explaining these differences between

chamber experiments and field include temperature gaps between air and spikelet, physiological status of the plant grown in field and pot.

GRAIN FILLING AND QUALITY

Effect of temperature on grain quality

High temperatures during ripening stages lead to smaller grain size and deterioration of grain appearance quality such as chalky grain and fissured grains. Under high temperatures, increase in grain weight is accelerated in the early ripening stages while duration of ripening period becomes shorter, which results in smaller final grain size (Sato and Inaba 1976). In recent years, high temperature above 26-27 ^{o}C during early ripening stages increase the occurrence of chalky grain and fissured grain in Japan (Fig. 1)(Kondo et al. 2006, Nagata et al. 2004, Terashima et al. 2001). Chalky grains are composed of various types such as white-backed, milky white, white-belly, and white-based grain having different opaque endosperm portion. Milky white grain seriously degrades the market value. Not only high temperature, but also other climatic conditions such as low radiation, typhoon and plant factors such as high spikelet number are important factor to cause milky-white grain.

Gain chalkiness is suggested to be caused by disorder of starch accumulation processes based on the fact that opaque portions of chalky grain, amyloplast development is abnormal (Zakaria et al 2002). The causes of chalkiness and small grain are hypothesized to be source limitation and/or disorder of starch synthesis processes. There are two major sources of carbon for starch synthesis in the endosperm, newly photosynthesized carbon from leaf and accumulated non-structural carbon, mainly starch and sugars, in stem and leaf sheath.

Photosynthetic rate at single leaf level is relatively stable under short-term exposure to wide range of temperature, e.g. 24 - 36 ^{o}C (Vong and Murata 1977).

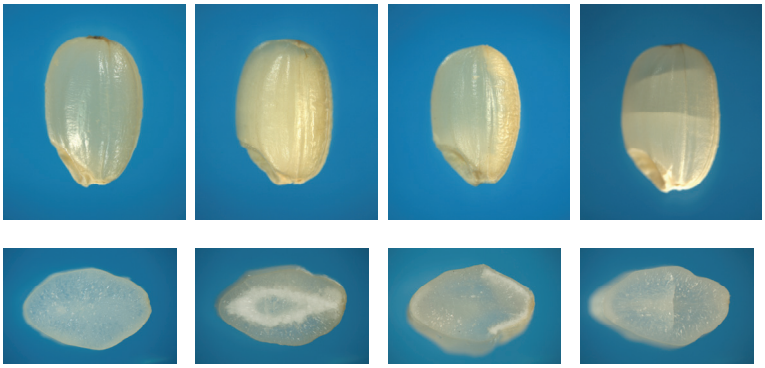

Fig. 1. Various types of grain appearance quality. From left: perfect grain, milky-white grain, white-backed grain, fissured grain. Photograph by N. Iwasawa.

The effect of high temperature on canopy photosynthesis under longer exposure to high temperature in field conditions is more complex since water and nutrient status of the plant are affected by temperature. The duration of active photosynthesis becomes shorter due to faster leaf senescence. During grain filling under higher temperature, decrease of non-structural carbon becomes faster, which indicate that carbon source for starch synthesis in endosperm depends more on reserved carbon in stem and leaf sheath to satisfy the increased carbon demand with accelerated grain filling. Further study is desired on the spatial and temporal substrate levels in endosperm as affected by temperature to clarify the involvement of limitation in substrate supply for starch synthesis.

The results in separate high temperature treatment on panicle and vegetative organs showed that treatment on panicle had more pronounced effect on final grain size and quality although vegetative organs were also sensitive to temperature to less extent (Sato and Inada 1973). It has been reported that activity and expression of some key enzymes involved in starch synthesis from ADP-glucose and conversion of sucrose to ADP-glucose are lowered under high temperature in cereal grains. In rice, high temperature depresses the expression of granule-bound starch synthase I, branching enzymes, and ADP-glucose

pyrophosphorylase while enhances expression of amylase (Yamakawa et al. 2007). Since the expression of genes and enzymic activity are affected by the substrate levels, the interaction of source and sink ability in response to high temperature should be further studied.

ADAPTIVE MEASURES

Cultivation methods

The agronomic countermeasures against high temperature damage include shifting cropping seasons, improving crop, soil, and nutrient management. Escaping from high temperature from anthesis and early ripening stage by moving transplanting date is the first major way in cropping system. The most sensitive period to high temperature is first 20 days after heading for chalky grains. Lowering expected temperature in this critical period by delaying of transplanting date has been successful to improve grain quality to some extent. Low N status magnifies the deterioration of grain quality, especially enhancing white-backed and white-base grains. Application rate of N has been decreasing since 1980s because of consumer's preference for low protein content in the grain. The low N application rate, in turn, induces the increased sensitivity to high temperature. Maintaining appropriate N status during ripening is important to maintain grain quality. Maintaining favorable soil conditions for root functions to ensure photosynthesis is important in soil management. Photosynthesis may be suppressed by high temperature due to reduced stomatal conductance, possibly related to the limitation of water uptake by root (Tsuno and Yamaguchi 1987). High soil temperature suppresses root growth (San-oh and Kondo 2006). Lowering soil temperature by cold water irrigation is effective to reduce fissured grain with the condition that water availability is not limited. The early drainage before harvest induces water stress that enhances deterioration of grain quality.

Varietal improvement in grain quality

Genotypic variations have been reported in sensitivities to high temperature in chalkiness and grain size. Genetic analyses for white-based and white-backed grains have been performed using japonica genotypes and several candidate QTLs were identified (Kobayashi et al. 2007, Tabata et al. 2007). Identification of the physiological functions of these QTLs is now under way. The genotypic differences in susceptibility to milky-white grains and grain size are less clear at present. Putative physiological and morphological traits related to the milky-white grain and grain size include stable sink ability under various temperature ranges, high source ability, low panicle temperature, and morphologies of the panicle and grain.

Future research

In physiological aspects, detailed understanding is required to clarify key carbon metabolisms which confer stable grain development under high temperature and low radiation. Genotypic improvement in adaptability to high temperature in spikelet formation, sterility and grain filling under are desired using wider germplasms. Limited information is available in genetic diversity in optimum temperature in grain filling and photosynthesis. Understanding the genetic factor and agronomic measure to control stomatal conductance would be useful because it determines canopy temperature and also affect water use. Increasing temperature is anticipated to have not only negative impact, but also positive aspects such as expanding crop growing spell. Development of crop production system which maximizes beneficial effect of global warming should be also considered.

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10~16

Changes in water distribution of developing rice caryopses by high-temperature stress

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ABSTRACT

High-temperature stress during grain ripening causes formation of chalky grains through loose packing of amyloplasts. The presence of chalky grains decreases the value of rice in most of the world markets. In the present study, the changes in water distribution in rice caryopses under high-temperature stress were investigated by magnetic resonance imaging. Milky-white and white-cored types of chalky grains, which had chalkiness around the centre of the endosperm, were frequently formed in high-temperature condition. Magnetic resonance images of the early-stage caryopses in high-temperature condition showed the lower water content around the centre of the endosperm, where the single-shaped amyloplasts was observed by scanning electron microscopy. In the middle stage, water content in the central chalky part became higher than in the lateral translucent part. Pooling of water in the free spaces between the loosely packed amyloplasts in the

chalky part led the higher water content after the middle stage. The physiological mechanism for the formation of white-cored and milky-white grains that occurred under the high-temperature stress was discussed in relation to these observations.
Keywords: Grain chalkiness, High-temperature stress, Magnetic resonance imaging, Rice, Water distribution

INTRODUCTION

Recently rice plants are often exposed to high temperatures during grain ripening in Japan possibly owing to global warming. The exposure to high temperature shortens the period for grain ripening but causes the formation of chalky grains. Considerable effort has been expended to understand the underlying mechanism of the high-temperature induced grain chalkiness from morphological (Zakaria *et al*., 2002), transcriptional (Yamakawa *et al*., 2007), proteomic (Lin *et al*., 2005) and genetic (Kobayashi *et al*., 2007; Tabata *et al*., 2007) perspectives.

During the ripening of cereal crops, a large amount of storage material accumulates in the seeds concomitantly with a decrease in water content. Magnetic resonance imaging (MRI) has led to the understanding of the precise water distribution in developing grains of barley (Kano *et al*., 1990; Glidewell, 2006) and rice (Horigane *et al*., 2001). However, these studies were conducted using caryopsis from normal condition; the relationship between change in spatial and temporal water distribution and the formation of grain chalkiness under high-temperature stress has not been studied yet.

In the present study, the water distribution in developing rice caryopses exposed to high-temperature stress was investigated by MRI, and its relation to the formation of grain chalkiness was discussed. Of the various types of chalky grains, we focused on the formation of the milky-white grains and white-cored grains, which were chalky in the central portion.

MATERIALS AND METHODS

Oryza sativa 'Koshihikari' (Japonica rice variety) was used. At 3 or 4 days after flowering (DAF), plants were transferred into a naturally illuminated temperature-controlled chamber. Day (13 h) and night (11 h) air temperatures were maintained at 26 °C and 20 °C in the control treatment, and 33 °C and 27 °C in the high-temperature treatment, respectively. Samples were collected three times (early, middle and late) during the dry-matter accumulating stage for each treatment. MRI was performed using an NMR spectrometer (DRX300WB, Bruker, Karlsruhe, Germany) equipped with a microimaging accessory at a magnetic field of 7.1 Tesla. The water distribution in cross-sections was estimated from proton density weighted (PDW) images. The in-plane resolution for the PDW images was 39 × 39 μm^2 and the slice thickness was 1 mm. At maturity, grain appearance was visually evaluated. Amyloplasts of perfect grains from the control treatment and white-cored/milky-white grains from the high-temperature treatment were examined by scanning electron microscopy (Real Surface View VE-7800, KEYENCE, Osaka, Japan).

RESULTS AND DISCUSSION

Perfect grains were predominantly formed in control condition, while over half of grains had white-cored and milky-white types of chalkiness in high temperature condition (Fig. 1A-D). Scanning electron microscopic observation of fully matured grains revealed that the starch granules were tightly packed in the grains from the control group (Fig. 2A). In contrast, starch granules were loosely packed and had single-rounded amyloplasts in the central chalky area of grains from the high-temperature group (Fig. 2B) as reported previously (Lisle et al., 2000). These results suggested that plastid initiation (i.e. amyloplast number) or starch granule initiation would be affected in the early dry-matter accumulating stage in high temperature condition.

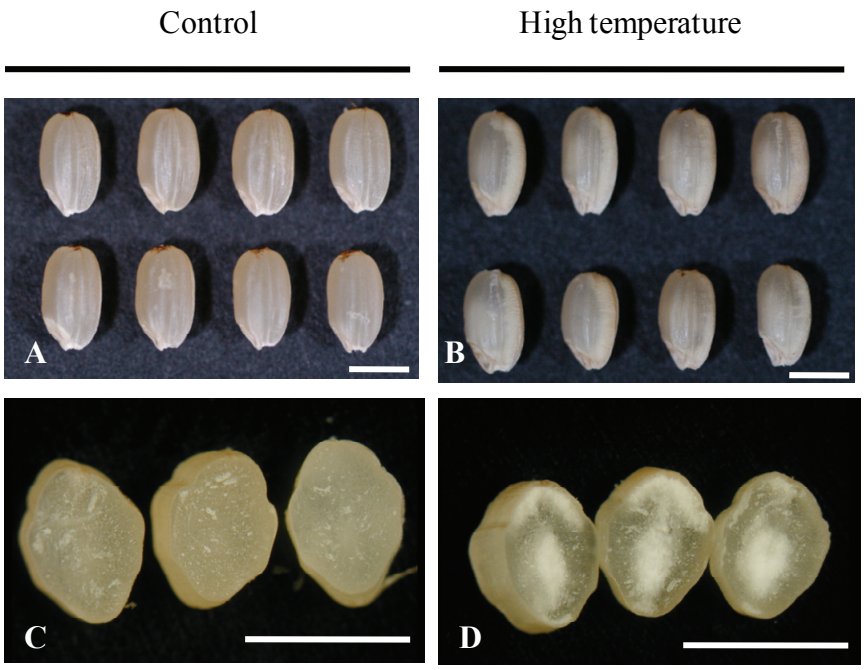

Fig. 1 Appearance of rice grains grown under control and high temperature condition. Typical appearance of grains (A, B) and median transverse sections of fully matured grains (C, D). Bar = 3mm

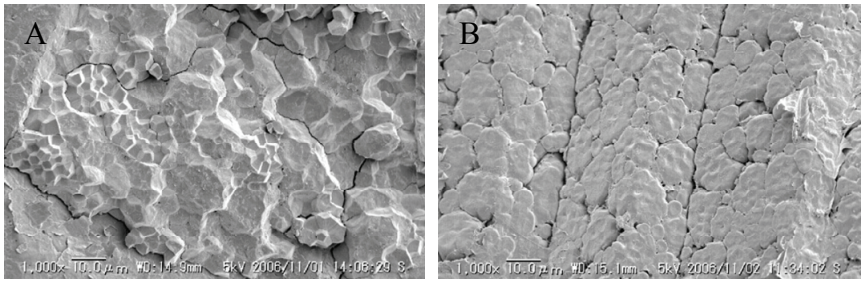

Fig. 2 Scanning electron micrographs of amyloplastss in the centre of matured grains from the control (A) and high-temperature (B) conditions.

Changes in water distribution in developing caryopses by high-temperature stress were investigated by MRI. In early dry-matter accumulating stage, the signal intensity was lower in grains from the high-temperature group than in those from controls especially along the dorso-ventral line (Fig. 3A, D), where coincided with the central chalky part of matured grains. This result suggested that the

lower water content of the central endosperm in the early stage of grain ripening in high-temperature condition might be related to the chalky centre through the formation of single-rounded amyloplasts. In contrast, the water content in the central core of the grain became higher in the high-temperature treated caryopses than in the controls in the middle stage (Fig. 3B, E). In the late stage, this pattern of water distribution was more clearly observed in the high-temperature treated caryopses than in the controls (Fig. 3C, F). Free water might have been pooled in the gaps between the loosely packed starch granules in the chalky area. This may be the reason for the higher water content in the core compared to the lateral parts for the high-temperature treated caryopses.

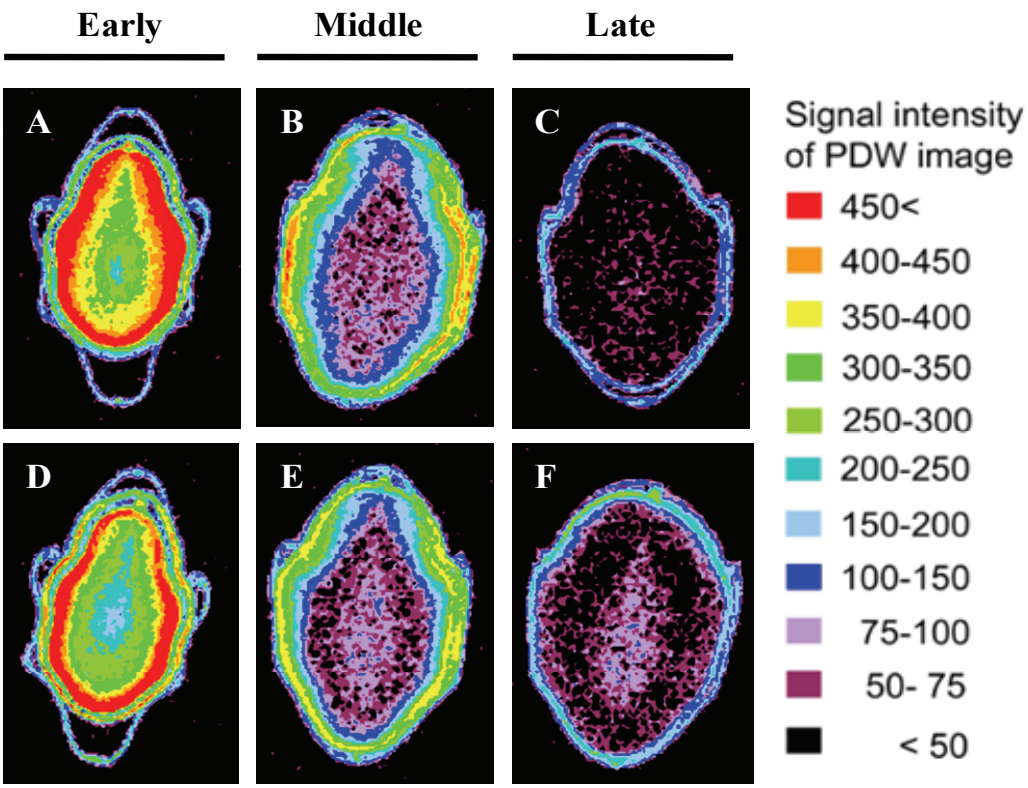

Fig. 3 PDW images of cross-sections of developing rice caryopses of controls (A, B, C) and high-temperature treated plants (D, E, F) in each dry-matter accumulating stage.

CONCLUSION

The present study clearly demonstrated the changes in water distribution of developing rice caryopses by high-temperature stress using MRI. The results indicated the possibility that disorganized formation of amyloplasts in the central endosperm of the grains from high temperature condition was due to rapid decline of water from early to middle stage. Further studies are needed to confirm our hypothesis that the water stress during the early stage of development is a cause of the hampered starch synthesis.

ACKNOWLEDGEMENT

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Fine Mapping of the Giant Embryo Gene *GE2* in Rice

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ABSTRACT

Giant embryo rice enriched with micro-nutrients can promote nutrition level of rice, and breeding of giant embryo rice is an important task. A new giant embryo variety, Tainung 78 developed by mutagenesis breeding, exhibits similar phenotypes of agronomic traits but 3~5 fold large embryo size to those of its derived parent, Tainung 72. After linkage analysis of 46 F_2 individuals of TNG 78 × TCS 17, the mutated gene conferring giant embryo was coarsely mapped in the interval between *RM243* and *RM420* on the long arm of chromosome 7, which was different position from the *GE*, and was temporally named as *GE2*. By employing additional 87 F_2 individuals and 5 markers to fine map *GE2*, the chromosome segment encompassing *GE2* was confined to 170 kb and contained 24 candidate genes after annotation. High resolution mapping by using more individuals and markers and sequencing the candidate genes are subjected to touch the target gene. Once *GE2* isolated via this positional cloning strategy, we can elucidate the mechanism of *GE2* how it enlarges embryo size under gene function and regulation. In the meanwhile, the two flanking markers of *GE2*, *CH0709* and *CH0720*, are subjected to rice breeding programs to breed more

giant embryo varieties. Furthermore, we are attempting to pyramid *GE2* and the golden rice gene of Tainung 76, *GR1* to purple rice CNY941201, to promote the nutrition of rice.

Key words: Giant embryo, Genetic mapping, Marker-assisted breeding, Rice, Tainung 78 (TNG 78).

INTRODUCTION

Rice, domesticated 9000 years ago, is the most important crop and is cultivated between 55°N and 36°S latitudes in various environments, such as irrigated, rainfed and floodprone ecosystems. There are two cultivated rice species, *Oryza glaberrima* cultivated only in Africa and *Oryza sativa* with two major subspecies, *japonica* and *indica*, widely cultivated mostly in Asian and other parts of the world (Khush 1997). More than three billion people in the world rely on rice, providing 23 % of calories consumed by human (Khush 2003). On the other hand, about 90% of rice is produced in Asia (Fisher *et al*. 2000), providing up to 35~60% of the total calories consumed by Asians (Khush and Brar 2002).

The edible endosperm contains carbonhydrate with majority which provides daily calories; on the other hand, rice embryo contains several micronutrients such as fatty acid, vitamins B and E, nicotana, and so on. Giant embryo with enlarged embryo size can promote the nutrition of rice. The giant embryo mutant of Kinmaze, induced by NMU (N-methyl-N-nitrosourea), enlarged embryo size of 2~3 fold embryo and increased the total fatty acid from 2.6% to 3.91% (Satoh and Omura 1981; Sato and Iwata 1990). Giant embryo rice with enlarged embryos enriches gamma-amino butyric acid (GABA) content in pre-germinated seeds (Yutaka *et al*. 1994), which is good for decreasing cholesterol and increasing antioxidants in vivo (Lee *et al*. 2007).

Three QTLs affecting embryo length mapped on chromosomes 1, 2, and 3, and three QTLs affecting embryo width mapped on chromosomes 2, 8, and 10, respectively, were identified from a *japonica/indica* cross (Dong *et al*. 2003). *GE*, a giant embryo locus with four mutant alleles (*ge-2*, *ge-3*, *ge-4*, and *ge-5*) induced by NMU, was reported and mapped on chromosome 7. The giant embryo was the consequence of enlarged scutellum and degenerated endosperm (Hong *et al*. 1995; 1996). Six giant embryo lines with 2~5 fold enlarged embryo size were selected and breed from *japonica* Tainung 72 induced by NMU (Wu and Lur 2002), and one of six was entitled as Tainung 78. In this study, we mapped the giant embryo locus of Tainung 78 by linkage analysis of genotypes of the giant embryo and molecular markers from segregating F_2 populations. The flanking markers of the giant embryo locus can be implemented marker-assisted selection in rice breeding to breed more elite cultivars possessing enlarged embryo.

MATERIALS AND METHODS

Plant Materials

The panicles of 20 individuals, after 10 hours of self pollination, of *Oryza sativa* ssp. *japonica* cv. Tainung 72, TNG 72, were soaked in 1 mM N-methyl-N-nitrosourea (NMU) for 4 hours; rice panicle were washed by running water about 6 hours and were planted in greenhouse to harvest M1 seeds in the first crop season, 2002. Approximate 4000 M1 were grown in paddy rice field of Chiayi Agricultural Experiment Station, Chiayi, Taiwan. The M1 plants were self pollinated, and three seeds of each M1 hill were randomly picked and bulked together. Approximate 4000 M2 individuals were planted in field in the first crop season 2003 and were screened for the desirable phenotype, giant embryo. Six M3 lines exhibiting giant embryo were consequently selected to self and propagate by pedigree method. The embryo size of the selected giant embryo

mutant line, entitled Tainung 78 (TNG 78), is about 3~5 fold larger than that of normal TNG 72 seeds.

To achieve the goal of fine mapping the mutated gene conferring giant embryo, the giant embryo variety TNG 78 was crossed to ssp. *indica* cv.Taichung Sen 17 (TCS 17), and the F_1 progenies were selfed to obtain F_2 seeds. About 46 and 600 F_2 individuals for coarse and fine genetic mapping were planted in paddy rice field of Chiayi Agricultural Experiment Station in the second crop seasons of 2007 and 2008, respectively.

Genetic Mapping of the Giant Embryo Gene

Molecular Marker Assay

The rice genomic DNA extraction was adopted the procedures of Li *et al.* (1995) with modification for mini-preparation. Approximately 0.02~0.1 gram of fresh tissues of 6- ~ 8-week-old young seedlings was homogenized in 900 μl of extraction buffer(100mM Tris, pH 8.0; 50 mM EDTA, pH 8.0; 500 mM NaCl; 1.25% SDS; fresh-made 0.38% of NaHSO3) at a frequency of 30 1/s for 2 min by TisseLyser (Qiagen, Germany). The homogenized leaf tissues were incubated at 65℃ for 30 min with twice gently inverted mix, and was subsequently added with 270 μl of 5 M KOAc, which was set on ice for 20 min. The supernatants were saved after centrifugation at 15,000 rpm for 10 min at 4℃, and 700 μl of ice-cold isopropanol was consequently added to precipitate DNA. DNA pellets were washed with 70% of ethanol, air dried, and dissolved in 100 μl of TE buffer.

Two types of public PCR-based markers, simple sequence repeat from Gramene (SSR; McCouch *et al.* 2002, IRGSP 2005) and sequence tagged site (STS) from Rice Genome Research Program, were first used to survey polymorphic markers between the parents, TNG 78 and TCS 17. If the linkage distances of two adjacent polymorphic markers were larger than 20 cM, we designed indel

markers, affixed by CH, SLS, and STS, based on blast genomic sequences of *japonica* cv Nipponbare version 5 against *indica* 9311. A polymorphic sequences with longer than 10 bp were selected and designed primers by using Primer3 on the basis of the flanking sequences of indels for PCR products of 100~300 bp after application. A total of 22 newly designed polymorphic markers were subjected to coarse genetic linkage analysis of *GE2* (Table 1).

PCR was performed in 25 μl of reactions containing 0.2 μ*M* of each primer, 200 μ*M* deoxyribonucleotides, 1× T*aq* buffer, 0.5 unit of GeneTaq DNA Polymerase (GenePure Tech, Taiwan), and 40-60 ng of rice genomic DNA. The PCR profile was: 94℃ for 5 min, followed by 35 cycles of 94℃ for 1 min, 55℃ for 1 min, 72℃ for 2 min, and finally by 5 min at 72℃ for the final extension (Biometra, German). PCR products subsequently were run on 2.5% agarose at 250V for 14~23 min, using FEBE electrophoresis system (Faster Easier Better Electrophoresis, Biokeystone, California, USA).

Genetic Mapping of the Giant Embryo Gene

A total of 84 DNA makers including 47 SSR, 15 STS, and 22 indel markers were applied to the F_2 segregating population of 46 F_2 individuals for coarse linkage mapping analysis. The genotype of the giant embryo gene of each F_2 individual was predicted by the segregation ratio of embryo size of its 50 seeds. The putative genotypes of giant embryo accompanied with genotypes of 84 markers were employed for linkage analysis, which was performed by the program MAPMAKER/EXP version 3.0 (Lander *et al*. 1987), using the Kosambi function (Kosambi 1944). The LOD threshold was fixed at 3.5, markers are unlinked while genetic distance more than 40 cM. For fine mapping, additional 87 F_2 individuals and five markers in the target region were applied to narrow down the chromosome segment encompassing the giant embryo gene.

Physical Mapping Analysis of the *GE2* Locus

To find out the physical position of *GE2*, the primer sequences of flanking markers were used as queries to search the *japonica* cv Nipponbare sequence database by BLASTN. The BAC/PAC contig encompassing were consequently identified, which was based on the rice pseudomolecules (release 5) based on the IRGSP minimum tilling path in representing the 12 rice chromosomes (IRGSP 2005) and was retrieved from Rice Genome Annotation (http://rice.plantbiology.msu.edu/)

RESULTS

Inheritance of Giant Embryo of TNG 78

The giant embryo variety, Tainung 78 (TNG 78), was derived from Tainung 72 induced by NMU and breed by pedigree selection. The agronomic traits of TNG 78, such as plant height, heading date, days to maturity, are similar to those of TNG 72. There is no significant difference in grain shape of grains and brown rice of TNG 72 and TNG 78, neither (Fig. 1). However, the embryo sizes of TNG 72 and TNG 78 are distinguishable that the embryo size of TNG 78 about 3~5 fold larger due to mutation (Fig. 1). The rice bran of TNG 78 including embryo contain enriched total of fatty acids, total of protein, minerals, amino acids, vitamin E, and other micronutrients (data not shown).

After six-year selection and purification, the maturity, morphologies and agronomic characters of TNG 78 resemble those of its derived parent, TNG 72, in field trials for the following years. TNG 72 exhibit enlarged embryo in every crop seasons, which implies that the inheritance of giant embryo of TNG 78 is stable. From the segregation ratio of normal to large embryo in the F_2 segregating population of TNG 78 × TCS 17 indicated the mutated allele resulting enlarged

Table 1. The newly designed polymorphic markers between Tainung 78 and Taichung Sen 17.

Primer	Chr	Position[a]	Forward sequence	Reverse sequence
STS322	1	10.9	AAGCTTTGACTTGTATTGCAC	TTTGTATCATGTTCCCAATTC
STS326	1	55.7	GCTCAGTATATTACGGTGGAA	GGGAAGTAAATTGAATTGGTT
STS420	1	157.6	ATTACCGACAGCAATAGTTCA	CACAGGTCATACACCATCTTT
STS307	2	118.1	GGCTGTTCCTACTTCCTAGTC	GCCTTATCCTGTACTTACACC
STS331	3	91.1	TGGGAGTGACAACTCATCTAC	ACACCAGCAAATCAGATACAT
SLS178	3	115.6	AGAAGAGTTACCTCCATCCT	AGGCTTCAAGTTATTGCTAA
SLS189	4	28.6	ATCAGAATATTCGGGAAAAG	TTGTATACTCATTATGTAAATGGA
STS332	4	97.4	ACAAACAGTTGTATCACTGGG	GAGAGCTAACGTCCTTGATTT
STS344	4	108.2	GTCGAGAGCAACACAATACA	TTAGATCATGATTCGGAAATG
STS230	5	67.5	TATATGGATCATCATGTGCAA	TCACAAACATCTGTGTGCAG
STS358	6	96.5	CTCATGACCCTCATAGAGCTT	TTAAGATGACATTAAATCACAC
SLS164	7	41.7	CTGCATATTTTCCCCTATTA	GGACAAGGCACTAATACAGT
CH0866	8	0.5	CTCCTTTCCCAATCTTACCT	GCGCATGCAGTATTATGTTA
SLS182	8	21.6-25.2	ATCCTGACCTCTTGTTCTAC	TTAACATAGAAGACCATACGC
SLS188	8	92.2	GGCATCAGTAAGACACAATA	AATGAATCTGTCTAGATTGG
CH0862	8	105.7-106.1	GAAGACGAGTGAGGTCAGAA	TCCAATAAAACTGAGGCTGT
SLS506	9	45.2-49.3	ATCTCTCTAATCTTGCTGGCT	TCCGTAACCCAAATAAACATA
SLS510	9	58.3-60.8	TCAATTTGTGGGTTAGGTTTA	AACTGTGATTATCAACACGC
CH1105	11	2.5-2.8	TCGTTTCCTTCAAAACCTTA	TTCGGTTGAACTGATAAATGT
CH1106	11	57.3	TGTTCTCTAGGGGAACAAAA	ATGCCTTTTTCGTAGGTGTA
SLS173	11	91.4	ACCCCTACCTCTACTAGTGC	CGGTTTGGGTGATAATATAG
SLS167	12	39.4	GCAATAAAAACATAAAAGCA	TAAAAATATGCTGAGCAGTC

a The linkage map position of the markers are referred to IRGSP after blasting its primer sequences against BACs.

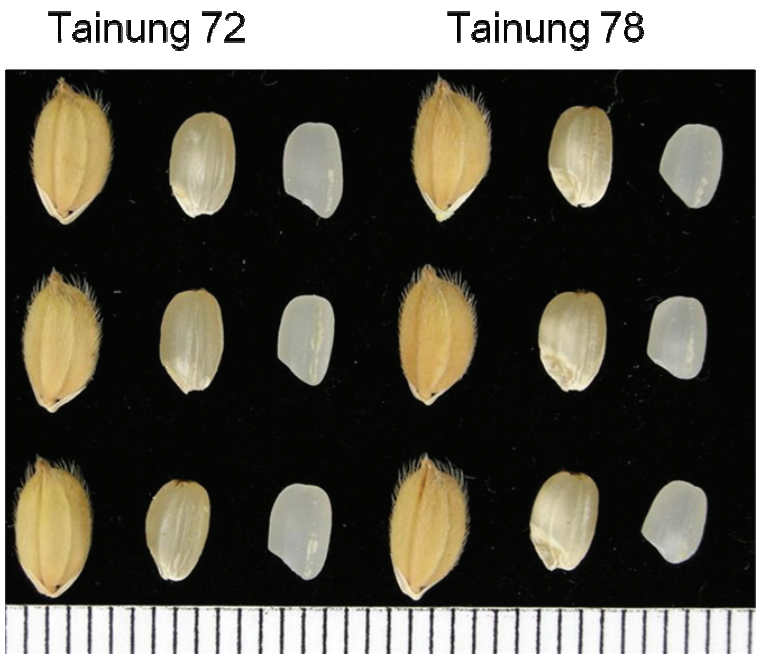

Fig. 1. The morphologies of seeds, brown rice and polished grains of TNG 72 (left) and TNG 78 (right).

Coarse Genetic Mapping of the *Giant Embryo 2*

A total of 84 PCR based markers, including 47 SSRs, 15 STS, and 22 indels, were subjected for coarse genetic mapping *Giant Embryo2, GE2*. Exclude chromosome 12 with one marker, the average interval of genetic distances of each chromosome ranged from 10.3 cM to 24.07 cM, with an average of 17.41 cM, based on the marker position aligned to the rice high-density linkage map published by Harushima *et al*. (1998) (Table 2).

Table 2. The markers employed to construct linkage analysis of *GE2*.

Chr	Genetic marker				Average genetic distance (cM)
	SSR	STS	Indel	subtotal	
1	5	3	3	11	20.41
2	9	0	1	10	17.09
3	4	3	2	9	15.12
4	3	1	3	7	17.35
5	4	2	1	7	14.80
6	6	0	1	7	17.71
7	5	1	1	7	24.07
8	3	1	4	8	18.53
9	2	2	2	6	19.68
10	3	1	0	4	10.30
11	3	1	3	7	16.44
12	0	0	1	1	-
Total	47	15	22	84	17.41

The coarse genetic linkage analysis was performed by 46 F_2 individuals of TNG 78 × TCS 17. The recombination frequencies of genotypes of 84 markers and the putative genotypes of *GE2*, which predicted by the segregation ratio of embryo size in approximate 50 F_3 seeds of each F_2 individuals, were analyzed by MAPMAKER/EXP version 3.0 (Lander *et al*. 1987) using the Kosambi function (Kosambi 1944). The *GE2* was resided in the interval of *RM234* and *RM420* on the long arm of chromosome 7, 9.0 cM away from *RM234* and 17.8 cM from *RM420* (Fig. 2).

Fine Genetic Mapping of *Giant Embryo2*

Additional 87 out of 600 F_2 of TNG 78 × TCS 17 planted in the 2nd crop season of 2008 were incorporated with five newly designed polymorphic markers in the target region to narrow down the chromosome segment encompassing *GE2*. *GE2*, flanking by *CH0709* and *CH0720*, is confined in an interval of approximate 170 kb (Fig. 2). The target region of *GE2* is covered by three BACs and encloses 20 genes, 3 putative/hypothetic genes, and one retrotransposon, annotated by MSU Rice Genome Annotation (Osa1) Release 6.1 (http://rice.plantbiology.msu.edu/cgi-bin/gbrowse/rice/).

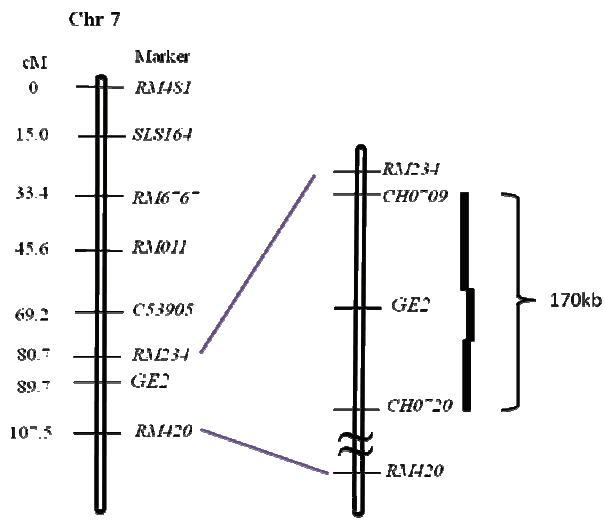

Fig. 2. The genetic and physical maps of *Giant embryo 2* (*GE2*).

DISCUSSION

Giant embryo rice enriched with total proteins, total fatty acids, vitamins, and GABA content in pre-germinated seeds. How to enlarge embryo size is an important task in rice breeding. It is not common to find giant embryo rice from natural germplasm because rice grains with large embryo are usually accompanied with small endosperm, leading to poor germination. Mutagenesis breeding by using chemical mutagens which results few mutations are adopted in giant embryo. A famous Japanese lowland rice cultivar with giant embryo, Hamiminori, was bred by the cross EM40 and Akenohoshi which EM40 was derived from Kinmaze induced by NMU (Hiroshi *et al*. 2001). Several new *indica* and *japonica* varieties were developed by mutagenesis in China, too (Zhang *et al*. 2007; Piao *et al*. 2009). In Taiwan, one of the authors, Y.-P. Wu who is a rice breeder, used NMU to induce TNG 72 and selected from 6 lines exhibiting giant embryo following by the pedigree method. One of six giant embryo mutant lines has 3~5 fold enlarged embryo was claimed as variety TNG 78 in 2009. TNG 78 possesses similar phenotypes of the agronomic traits to its derived parent TNG 72, such as plant height, heading date, yield components, and aroma (Fig. 1). As the high potential nutrition of giant embryo rice, TNG 78 can be utilized in enriching human nutrition as a daily food and as nutrition supplements by brown rice tea bag, healthy nutrition addition agents, and germinated rice.

The locus of *GE2* was coarsely mapped in the interval 26.8 cM, between *RM234* and *RM420* on the long arm of chromosome 7, corresponding to the interval of 14.4 cM from 93.9 cM (*RM234*) and 118.3 cM (*RM420*) on the long arm of chromosome 7, with an interval of 14.4 cM, based on the rice high-density linkage map published by Harushima *et al.* (1998) (Fig. 2). The discrepancy in

interval lengths of *RM234* and *RM420* is because of different numbers of F_2 individuals and different parents used in crosses. The alternative possibility was 46 individuals subjected to coarse mapping were pre-selected for giant embryo phenotype, leading to higher local recombination rates surrounding *GE2*. The locus of *GE* with four alleles was mapped at the interval of 86.7 cM and 93.0 cM of chromosome 7 by means of high resolution mapping and physical mapping (Nagasawa *et al*. 2002). The linkage maps of *GE* and *GE2* are slightly different that *GE2* might be a new locus corresponding to enlarged embryo size.

GE2 was fine mapped in a 170-kb chromosome segment by recombination study of additional 87 F_2 individuals and 5 markers. The candidate genes were delimited to 20 genes and 3 hypothetic/putative genes. Further investigations of high-resolution mapping by more recombinants and high-density markers to confine the target region with less candidate genes, consequently on positional cloning of the isolation gene, are being carried out to elucidate the gene function and regulation of *GE2*. The 'alteration of plant embryo/endosperm size during seed development', including giant embryo *GE* with four alleles, is patented (WO/2007/070687, 2007; US7,582,817 B2, Sep. 1, 2009). Thus, the flanking markers of *GE2*, *CH0709* and *CH0720*, can be implemented to rice breeding programs to breed more varieties with enlarged embryo by marker assisted selection as foreground selection.

In addition, the flanked markers of the giant embryo can be incorporated with the functional marker of golden rice gene *GR1* of *japonica* Tainung 76, a sodium azide derived from *japonica* Tainung 67, to introgress into a purple pericarp line, CNY941201, to promote nutrition of rice which can alleviate malnutrition as rice for the major staple food.

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Marker-assisted selection for biotic stress tolerance in rice: current status and emerging needs

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Introduction

Because of the intensive studies done on host plant resistance and host-pathogen interactions for some rice pathosystems, many useful resistance genes and markers are available in rice. This makes it possible to practice marker-assisted selection (MAS) for disease resistance breeding at a relatively early stage. Beside scientific advances, there are also practical reasons why MAS is particularly relevant for breeding for biotic stress tolerance:

- Most disease assays require the use of pathogens and insects, which are often variable and contribute considerable variation in the phenotypic evaluation. In some cases, the pathogens and insects as screening agents are geographically limited due to quarantine measures.
- Interactions between host and pathogen often exhibit strong epistasis; i.e. resistance tends to mask the phenotypic expression of other genes. Thus, gene pyramiding is possible only through the use of genetic markers.
- In the case of insect-transmitted viruses, phenotypic evaluation is further complicated by the complex interactions of host, vectors, and viruses.

These limitations can be addressed by using genetic markers as a surrogate for the target phenotype. Thus, MAS is used not only as a tool to increase efficiency but an essential approach for achieving a breeding objective. However, no MAS will be successful if the genes/markers do not represent high-quality genes expressing

the appropriate spectrum of resistance. In this brief review, we will discuss the current status of MAS applications for key disease and insect pests of rice, the on-going needs, and emerging challenges.

Bacterial blight resistance: a successful case of MAS in rice breeding

MAS in bacterial blight (BB) is the most advanced in rice breeding program, both in terms of science and in producing commercial products. A number of releases with different *Xa* combinations have been made in the past decade. The pathway to success dated back to over 20 years ago when a concerted effort was made to produce a comprehensive series of near-isogenic lines for all bacterial blight resistance genes (*Xa*). In retrospect, the key features for success may include the following:

- A strong research environment exists for studying host-pathogen interactions of bacterial blight, leading to many mapped and cloned *Xa* genes, hence providing either very tightly linked or gene-based markers. A list of widely used genes is summarized in Leung (2008).
- Most of the discovered *Xa* genes are bred into the indica recurrent parent IR24, a variety with wide adaptability and high agronomic qualities. This breeding-ready background accounts for the extensive use of *Xa* genes in breeding programs of both the public and private sectors. Recurrent parents in japonica (Toyonishiki) and indica-japonica (Milyang 23) backgrounds were also made.
- Extensive testing data on individual *Xa* genes and gene pyramids across locations.
- A capacity building network sustained continuously for 9 years by the Asian Rice Biotechnology Network. It facilitated distribution of NILs and gene pyramids, provided training and capacity building, and on-site technical backstopping to breeding programs.

These positive attributes appear simple, yet establishing such a system requires an understanding of the biology of the pathosystem, systematic development of specialized genetic stocks, and a network to evaluate and disseminate the genes/breeding lines and appropriate diagnostic BB strains.

Yet, even with this successful record, bacterial blight has not disappeared as a problem. There is a continuing need for different combinations of *Xa* genes to cope with new virulences and changing production system. This is particularly evident in India and China. Bacterial blight is a key problem in the fast expanding lowland rice production in western Africa. Because of the generally low level of resistance in many hybrid rice varieties, bacterial blight is expected to become more prominent with the spread of hybrid rice technology. This will require an increased effort to incorporate *Xa* genes into parental lines for both 3-way (CMS line) (Borines et al, 2008; Agarcio et al, 2007) and 2-line hybrids (Perez et al. 2008).

Blast

Just like the *Xa* genes, many blast resistance genes *Pi* have been reported in the literature. However, the use of MAS in blast resistance breeding has lagged behind bacterial blight. There are at least two reasons for this gap. First, breeding for blast resistance cannot follow the simple pyramiding of major R gene as in bacterial blight. The blast fungus appears to have a dynamic genome with great capacity to change in response to R genes deployed in rice varieties. Consequently, breeding for blast resistance requires a diversity of *Pi* genes and QTL. Second, many *Pi* genes have been bred into the genetic backgrounds of Co39 (indica type) and LTH (japonica type) because of their wide-susceptibility to blast (Fukuta et al 2004, Kobayashi et al, 2007; Fukuta et al, 2009). This comprehensive series of NILs and monogenic lines are ideal materials for genetic studies but less so for varietal development because they do not have the wide agronomic adaptability, such as IR24.

Table 1. *Pi*-genes used in breeding program of NARES partners.

Pi-gene	Designation	Donor	Chro	Class of gene	Near-isogenic line			Being used in breeding
					LTH	CO-39	US-2	
Piz	IRBLz-Fu	Fukunishiki ML: IRBLz-Fu	6	-	BC3F1 -	- -	- BC4F1	-
Pish	IRBLsh-S	Shin 2 MlL: IRBLsh-S	1	-	- BC3F1	BC6F14 BC4F1	- BC3F1	Philippines
Pi1	IRBL1-CL (C101LAC)	C101LAC ML:IRBL1-CL (C101LAC)	11	-	BC6F16 -	BC6F14 -	- BC6F7	Indonesia; India
Pi3	IRBL3-CP4 (C104PKT)	Pai-kan-tao ML: IRBL3-CP4 (C104PKT) Taebeg	9	-	BC6F16 - BC6F15	- BC4F1 -	- BC4F1 -	Vietnam
Piz-5 = Pi2	IRBLz-CA (C101A51)	C101A51 ML: IRBLz-CA	6	NBS-LRR (*Nbs4-Pi2*)	BC6F16 -	BC6F14 -	- BC4F1	Indonesia
Pi5(t)	IRBL5-M	RIL249 (Moroberekan) ML:IRBL5-M (RIL249)	9	-	BC6F16 -	BC6F14 -	- BC6F8	-
Pi9(t)a	IRBL9-W	Oryza minuta WHD-IS-75-1-127 ML:IRBL9-W (WHD-IS-75-1-127)	6	NBS-LRR (*Nbs2-Pi9*)	BC6F16 -	- BC3F1	- BC6F7	India
Pik-p	IRBLkp-K60	HR 22 K60 ML:IRBLkp-K60 (K60)	11	-	- BC3F1	BC6F14 -	- BC6F7	-
Pik-h	IRBLkh-K3	HR 22 K3 ML: IRBLkh-K3 (K3)	11	-	BC6F14 -	BC6F14 -	- BC6F7	Bangladesh
pi21	-	Owarihatamochi		Proline-rich protein	-	-	-	Japan
Pi36	-	Kasalath	8	Rice coiled-coil-NBS-LRR, resembles *Mla1* and *Mla6* than *Pita, Pib, Pi9*, and *Piz-t*.	-	-	None	None; evaluated against Chinese isolates
Pi40(t)	IR65482-4-136-2-2b	*O. australiensis* (Acc. 100882)	6	NBS-LRR motifs	-	-	-	-

Recent developments in blast research provide new opportunities to implement MAS in blast resistance breeding:

1. Increasing amount of experimental data has enabled us to distinguish high-quality *Pi* genes (Table 1). Some of these genes have been cloned, hence providing gene-based markers for use (Liu et al. 2009).
2. Blast resistance QTL are available that can provide broad-spectrum resistance—e.g. germin and germin-like protein, complex gene loci together give QTL effect (Manosalva et al, 2009; Carrillo et al, 2009).
3. Novel genes conferring broad-spectrum disease resistance have been identified. One such example is *pi21*, a resistance gene that provides stable blast resistance in upland rice grown in Japan. (Fukuoka et al, 2009). Unlike the "traditional" R genes with NBS-LRR domains, the wild type *Pi21* gene encodes a proline-containing protein such that a recessive mutation of the gene confers partial resistance. It would be interesting to introduce *pi21* into multiple genetic backgrounds and agronomic settings.

Because gene-based markers are available, production of gene pyramids can be accelerated by marker-assisted selection and without total dependency on phenotypic evaluation. It means that genotypes can be constructed with combinations of multiple R genes and QTL.

Brown Planthoppers (BPH)

Application of MAS for selection of insect resistance is most advanced for gall midge because of the strong interaction phenotypes between gall midge and rice. The observations so far suggest a gene-for-gene interaction similar to that found in highly specialized host-pathogen interactions. For tropical Asia, the key insect problem amenable to a MAS breeding approach is brown planthopper because the number of genes identified for BPH is high (Yencho et al, 2000; Jena et al, 2006; Rahman et al, 2009). Recent study has evaluated the virulence of laboratory strains of the BPH (*Nilaparvata lugens*) and the whiteback planthoppers

(*Sogatella furcifera*) collected between 1966 and 2005 using rice differential varieties carrying different planthopper resistance genes. The study suggested that long-term mass rearing in the laboratory has not affected virulence status, thus will be useful in analyzing resistance genes against BPH and WBPH (Myint et al, 2009).

Resistance from wild rices has become the potential for exploring novel genes. Of 21 BPH resistance loci, 11 have been identified from wild rice species; they are *Bph10* (*O. australiensis*), *Bph12(t)* (*O. latifolia*), *Bph13(t)* in chromosome 2 (*O. eichingeri*) and another *Bph13(t)* in chromosome 3 (*O. officinalis*), *Bph14* and *Bph15* (*O. officinalis*), *Bph18(t)* (*O. australiensis*), *bph11(t)* and *bph12(t)* (*O. officinalis*), *Bph20(t)* and *Bph21(t)* (*O. minuta*). Major genes conferring BPH resistance in several cultivated rice and wild species have been mapped with markers that will facilitate MAS for BPH resistance (Jena et al, 2006). Also, there is an on-going effort to produce series of NILs carrying important *Bph* resistance genes.

We foresee a growing need for MAS for insect resistance breeding. For example, the donor cultivar Rathu Heenati carrying *Bph3* was combined with KDML105 essential grain quality traits. The linkage drag between *Bph3* and Wx^a alleles was separated by phenotypic and MAS (Jairin et al, 2009). All introgression lines from this population showed broad spectrum resistance against BPH populations in Thailand and had grain quality similar to KDML105.

Tungro viruses

Since the 1990s, a number of tungro resistant varieties has been developed by direct phenotypic screening. Initially, most selected varieties were resistant to the green leafhopper (GLH). Emphasis was later shifted to resistance to tungro viruses (e.g., the development of Matatag 1). However, there is difficulty in conducting screening because the phenotypic assay is often based on the percent of plants showing symptom expression within a line. The complexity of the

interactions involving three biological agents: GLH harboring two different viruses—RTSV and RTBV makes tungro virus resistance an excellent target for MAS application.

To identify gene(s) involved in RTSV resistance, the association of genotypic and phenotypic variations for RTSV resistance was examined in backcross populations derived from Utri Merah and rice germplasm with known RTSV resistance. Resistance in Utri Merah was found to be under the control of a recessive gene (*tsv1*). The gene was mapped to a 200-kb region (22.0 to 22.2Mb) of chromosome 7. Within this region, Lee et al (2009) identified SNP variants in a gene encoding initiation elongation factor (IEF) on chromosome 7 that is strongly associated with RSTV resistance. Donors and germplasm showing resistance all have the same SNP variants affecting the Val variants in the IEF gene. Thus, the evidence so far suggest that mutations in the IEF could be responsible for resistance to RTSV, and the SNP variants could serve as markers for selection for RTSV resistance.

Sheath blight

Although there have been increasing number of reports on the mapping of QTL for sheath blight resistance, most of them are coarsely mapped, spanning a large genetic distance. Furthermore, the phenotypic effects of these QTL are relatively small. This level of phenotypic effects does not inspire the use of the QTL by breeders due to the inherent difficulty in assessing the components of resistance associated with disease progression. In a recent study, 12 QTLs were identified for sheath blight resistance on the HP2216/Tetep recombinant inbred population from a cross between HP2216 and Tetep. These QTLs on chromosomes 1, 3, 7, 8, 9 and 11 and the respective alleles explain 8.13-26.05% of the total phenotypic variation (Channamallikarjuna et al, 2009). Progress has also been made in identifying good sources of genetic resistance using standard evaluation

procedure for sheath blight resistance (IRRI SES, 1996). We evaluated over 300 accessions of *Oryza rufipogon.* Of these, three accessions were selected and backcrossed to elite lines and varieties. Advanced F_4 population derived from the cross *O. rufipogon* (Acc 105757) x PSBRc80 have been selected for further backcrossing and intermating to develop populations for resistance breeding.

Effort is also underway to refine the phenotyping procedures. Until we have confidence in the assay of sheath blight resistance at different levels (tillers, single hill, to plant population), it will be difficult to develop molecular markers with high predictive power on the phenotype.

Brown spot

Relatively little is known about the genetic control of resistance to brown spot. Significant differences in resistance were observed in a diverse set of germplasm tested against isolates of *B. oryzae*. Among the germplasm tested, N22, MAAL 6, IR36 and restorer line IR69726-29-1-2-2-2 showed variation in resistance to different isolates of the pathogen.

Of special interest is Dinorado (IRTP 12568) which shows resistance to multiple virulent isolates of *B. oryzae*. To determine the resistance in this tall traditional japonica variety, a mapping population was developed by crossing Dinorado with IR36, a susceptible indica variety. Based on segregation of F_2 and F_3 progenies, two pairs of recessive genes appeared to be responsible for the resistance to brown spot. Based on inheritance study, the two genes are tentatively designated as *bs1* and *bs2*.

A QTL was defined by markers RM277 and RM1261 on chromosome 12, which accounts for 48% of total phenotypic variation. The second QTL was mapped on chromosome 3 with lesser effect. Further fine mapping of these two QTL may yield markers useful for MAS.

Conclusions

For diseases such as bacterial blight and blast where resistance genes have been identified and their quality assessed, incorporation in improved high yielding varieties have been made and released for commercial production. An intensive knowledge of host-pathogen interaction for diseases with specific resistance contributes to identification of good quality genes that confer broad spectrum resistance. The mapping and cloning of well-studied genes allows identification of tightly linked markers as well as design of gene-based markers for the breeding program. For quantitative traits, testing in multi-location sites for studying GxExM interactions allows for precise phenotyping of the target traits in response to the diverse population of pathogen and insect pests. Despite these challenges, genetic resources and tools have been developed for biotic stress tolerance in rice (Table 2) and genetic stocks with value-added traits are available for some traits, and for other traits are still in progress (Table 3). Efficiency in utilizing these genetic resources to develop and sustain durable resistance in agricultural system is needed.

Under an environment influenced by drivers of global changes in rice production (climate, labor, water scarcity, etc), impacts on production technologies become a concern. Diseases such as false smut and those causing grain discolorations are emerging problems in rice production that need to be addressed in the future. These are concerns closely associated with long term sustainability and environmental consequences of intensification of agricultural systems.

Table 2. Genetic resources and tools for biotic stress tolerance.

Biotic stress	Conventional breeding	MAS Component			Genes/QTL transferred into new backgrounds	MAS products (commercial)	Trans genes or GM
		Genes/QTL mapping	Candidate genes	Gene discovery			
BB	+++	+++	-	-	+++	+++ (released)	+
Blast	+++	+++	+++	++	+++	-	-
Tungro	+++	+++	+	++	+++	+	+
Sheath blight	+	+++	-	-	-	-	++
Brown spot	+	+	-	-	-	-	-
False smut (emerging)	-	-	-	-	-	-	-
BPH	++	+++	-	-	+	-	-
WBPH	+	+	-	-	-	-	-
Stemborer	+	+	-	-	++	-	+++

Table 3. Genetic stocks with value-added traits for biotic stress tolerance.

Trait	Genetic stocks
Bacterial blight	Near-isogenic lines (IR24, Toyonishiki, Milyang 23) MAS pyramided lines (IR24) Alien introgression lines
Blast	Near-isogenic lines (*Pi* genes & candidate genes in 2-3 backgrounds, in progress) Alien introgression lines
Tungro	Elite tungro resistant lines Alien introgression lines
Sheath blight	Genetic donor Mapping population (in progress)
Brown spot	Genetic donor Mapping population (in progress)
BPH	Elite breeding lines Alien introgression lines
WBPH	Genetic donor Elite WBPH resistant lines
Stemborer	Donors Elite stemborer tolerant lines

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Marker-Assisted Breeding for Abiotic Stress Tolerance in Rice: Progress and Future Perspectives

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ABSTRACT

While global climate change threatens to increase the occurrence and intensity of production constraints such as abiotic stresses, new marker-assisted breeding techniques may enable the rapid development of stress-tolerant rice varieties to face these challenges. Through the mapping of quantitative trait loci (QTLs) and precise transfer of tolerant QTLs by marker-assisted backcrossing (MABC), improved varieties can be developed with beneficial traits. For example, salt stress is a major constraint across many rice-producing areas because of the high sensitivity of modern rice varieties. At IRRI, we are working towards combining QTLs conferring salt tolerance to develop robust cultivars for stress-prone environments. Previously, RILs from a cross between IR29 and an accession of the tolerant landrace Pokkali were used to map several salt tolerance QTLs, including *Saltol*, a major QTL on the short arm of chromosome 1. Near-isogenic lines were developed between IR29 and Pokkali, and subsequently multiple Pokkali alleles were identified between the original RILs and the NILs. The Pokkali introgressions in six advanced lines were also characterized using an Illumina GoldenGate assay developed at Cornell University to genotype 1,536 SNP loci across the rice genome. A precision marker-assisted backcrossing

system is also being employed to efficiently transfer beneficial Pokkali introgressions into popular varieties such as BRRI dhan28, an important dry season variety in coastal Bangladesh, to increase production in saline prone areas. Future advances in new marker-assisted breeding strategies, rapid sequencing of multiple rice genomes, and SNP genotyping technologies also promise to increase the efficiency of marker development and integration of molecular tools into modern breeding programs.
Key words: *Orzya sativa*, Molecular breeding, Salinity tolerance, SNP genotyping.

INTRODUCTION

Although plant breeders have made significant progress using conventional breeding methods, the past 20 years have seen the potential of molecular markers gain prominence in breeding programs. Even though the types of molecular markers have changed over the years, the basic concept is the same: markers allow tracking of polymorphisms based on underlying changes in the DNA of each individual. These markers can then provide information on genetic relatedness, linkage to important traits, and detection of donor introgressions in segregating populations. Ultimately, the usefulness of integrating molecular markers into a breeding program will depend on the how well the markers can replace phenotyping, both in terms of cost-effectiveness and in their ability to predict performance under different environments. Although great efforts have been invested in mapping genes and in developing marker techniques, only recently have markers become routinely used in major breeding programs, as evidenced by the successful release of the first generation of marker-assisted breeding products from both the public and private sector. There are a number of strategies now available to incorporate molecular tools into a rice breeding program to increase the efficiency and power of selection, as described below.

Mapping Quantitative Trait Loci (QTLs)

While early success was made with mapping genes for qualitative traits, many traits in rice breeding are quantitative traits showing a normal distribution, due to control by multiple genes and environmental interactions. Mapping QTLs, which are chromosomal regions that contain a gene or genes contributing to a trait phenotype, requires comprehensive genetic maps to assign a phenotypic effect at markers near the QTL. Numerous QTL studies have led to an explosion in the number of QTLs mapped in rice for a multitude of traits, with over 8,000 QTLs now available in the online Gramene database (www.gramene.org). While there are dozens of QTLs mapped for each major trait, the successful integration of QTLs into MAS programs has been more elusive, likely due to the fact that many traits are controlled by multiple small effect QTLs, often complicated by environmental interactions and/or differing genetic background effects. Care must be taken to select the most promising QTL targets for breeding applications, focusing on large effect QTLs that are stable across environments and genetic backgrounds.

Exploring Novel Genetic Variation

The basis for future improvement in breeding programs is the successful identification and use of novel genetic variation. Much work has been done to characterize the genetic diversity across rice varieties on a global scale, but more research is needed to investigate diversity in more detail on the national and local scales. For example, the genetic diversity of traditional rice varieties in Indonesia revealed important differences between representative rice accessions across a geographically diverse sample versus newly collected varieties in an isolated region in Borneo (Thomson et al. 2007; Thomson et al. 2009). While it can be difficult to identify and extract useful variation directly from germplasm collections, one advantage of QTL mapping is that it can enable the discovery of

hidden diversity in exotic germplasm that is not readily accessible to breeders (McCouch et al. 2007). For example, a QTL study between a U.S. variety and an accession of *Orzya rufipogon* identified transgressive QTLs from the wild rice relative that could improve the elite culitvar (Thomson et al. 2003). A large investment is often required to further characterize target QTLs through near-isogenic line development and subsequent fine-mapping, as was performed for *O. rufipogon*-derived QTLs for grain weight, heading date, and red pericarp (Li et al. 2004; Thomson et al. 2006; Sweeney et al. 2006).

Marker-Assisted Backcrossing (MABC)

One of the simplest forms of marker-assisted breeding is the use of molecular markers to improve the conventional backcross conversion method, where the desired trait is transferred to another line through crossing followed by repeated backcrossing to the recurrent parent to reconstitute the original variety. The use of markers can speed up the conversion process and greatly reduce the amount of negative linkage drag correlated with the target locus. Consequently, the technique of marker-assisted backcrossing (MABC) has been promoted as a way to take beneficial QTLs and incorporate them into breeding lines using foreground markers at the target locus and background markers across the rest of the genome. Thus the new paradigm for non-transgenic molecular breeding follows a pathway from identifying landraces as donors for the trait of interest, to QTL discovery and fine-mapping of the most promising loci providing tolerance, and finally using marker-assisted backcrossing to rapidly transfer the QTLs to popular varieties. Recently, the power and efficacy of this approach was proven through the discovery, fine-mapping and cloning the submergence tolerance QTL *Sub1* from the tolerant landrace FR13A, followed by successful MABC transfer of *Sub1* into the popular variety Swarna and five other mega-varieties (Xu et al. 2006; Neeraja et al. 2007; Septiningsih et al. 2009). In practice, the efficiency

of a MABC program depends on a number of factors, including the availability of markers in the target region, the precision of the target gene/QTL fine-map, the rate of polymorphism when identifying background markers, and the cost, speed, and failure rate of the markers employed in each customized MABC system.

Pyramiding Genes and QTLs

The use of molecular markers can also enable breeders to pyramid, or combine, multiple genes or QTLs into the same variety. In some cases, such as the pyramiding of resistance loci conferring resistance to a disease, relying on phenotype alone would be impossible, but using markers allows the tracking and combination of specific resistance genes. Pyramiding can also be used to "stack" genes for multiple traits into the same variety. For example, gene pyramiding has been successfully used in rice to combine multiple genes for bacterial leaf blight, as well as resistance genes for other pests and diseases (for review, see Collard and Mackill, 2008). When dealing with quantitative traits, such as abiotic stress tolerance, QTL pyramiding as been proposed as a strategy to combine multiple QTLs, each controlling a trait by a different mechanism, to increase the contribution to the trait effect and for developing optimal combinations of QTLs for multiple traits.

Gene Discovery and Functional Markers

Although foreground selection using closely linked markers for major genes and QTLs is the most common approach for marker-assisted breeding, a refinement of this strategy is to target the genes themselves for marker development. Intense research efforts around the world are focused on dissecting the molecular mechanisms and genetic pathways underlying key traits in rice—leveraging the value of the complete genome sequence for gene discovery and functional analysis. One common justification of the massive research investment in rice

functional genomics is that the end result will empower more efficient strategies for molecular breeding. Thus genomics-assisted breeding promises to apply discoveries from mutant analysis, DNA microarrays, QTL cloning and allele mining to develop more precise molecular tools for crop improvement (Varshney et al. 2005; Leung 2008). The most intuitive way to do this is to target a functional nucleotide polymorphism, which is a change in a gene that causes the desired phenotype, for development of functional markers (Andersen and Lubberstedt, 2003). By relying directly on the causal polymorphism, these "perfect" markers will be diagnostic of the favorable allele, since they will always co-segregate with the trait phenotype. For example, once the gene controlling aroma in rice was cloned, the sequence polymorphisms that led to increased aroma were identified and developed into a perfect marker for fragrance in rice (Bradbury et al. 2005). As more genes controlling key traits in rice are characterized, there will be more opportunities to mine superior alleles from germplasm collections and to develop functional markers for more precise and efficient marker-assisted selection.

RESULTS AND DISCUSSION

Breeding for Salinity Tolerance in Rice

Salinity is a problem in coastal regions due to intrusion of brackish water during the dry season and a worsening problem in inland areas with saline soil and/or poor quality irrigation water. There are a number of traditional varieties adapted to these regions which show a remarkable tolerance to salinity and have been used to study the basis of tolerance in rice. Tolerance to salinity may involve a number of different physiological mechanisms, including sodium exclusion from roots, controlled sodium transport between root and shoot, sequestering of sodium in older tissues and in the vacuoles, better stomatal control, and antioxidant activity (Ismail et al. 2007). As traditional landraces have shown tolerance

through different mechanisms, a pyramiding approach has been proposed to combine complementary mechanisms to develop rice varieties with improved levels of tolerance. Progress has been made to transfer salinity tolerance from traditional donors to modern high yielding varieties, but the complexity of the trait has proven challenging to breeders. Since most of the tolerant landraces are low-yielding varieties with numerous undesirable traits, a precision marker-assisted breeding approach is needed to rapidly transfer the high levels of tolerance of the landraces into high yielding breeding lines while at the same time avoiding negative linkage drag.

QTL Mapping and MABC for Salinity Tolerance

Previously, a recombinant inbred line (RIL) mapping population between IR29 and the salt tolerant landrace Pokkali was used to identify a major QTL for seedling-stage salinity tolerance, named *Saltol*, on the short arm of chromosome 1, along with a number of minor QTLs. Subsequently, RFLP and SSR markers were added to the *Saltol* region to more precisely define the position (Bonilla et al. 2002). Near-isogenic lines were developed between IR29 and Pokkali, and multiple Pokkali alleles were identified between the original RILs and the NILs. The Pokkali introgressions in six advanced lines were also characterized using an Illumina GoldenGate assay developed by Susan McCouch at Cornell University to genotype 1,536 SNP loci across the rice genome. Other Pokkali QTLs are being targeted for NIL development to allow testing of individual QTL effects. Furthermore, mapping populations using additional sources of tolerance are also being developed at IRRI to compare QTLs across different tolerant donors under both seedling and reproductive stages. A precision marker-assisted backcrossing system is also being employed to efficiently transfer beneficial Pokkali introgressions into popular varieties such as BRRI dhan28, an important dry season variety in coastal Bangladesh. These new salt tolerant varieties can then

help increase production in stress-prone environments, which will likely pose increasingly difficult growing conditions in the future.

CONCLUSIONS

Future Perspectives

Advances in genomics promise to enable more powerful trait dissection through techniques such as global genome expression analysis, rapid re-sequencing of whole genomes, and association studies using diverse germplasm (Zhu et al. 2008). This wealth of data can be combined with QTL analysis using a positional candidate gene approach to identify converging lines of evidence pointing towards the key genetic players that control abiotic stress tolerance pathways. Moreover, knowledge of the genetic pathways can be integrated with a detailed physiological analysis of tolerance mechanisms to achieve a more complete picture of the rice plant's response to salinity at the whole plant level. Although QTL discovery, fine-mapping, and marker-assisted backcrossing is a straightforward and powerful technique, there are still many challenges to be faced when applying this approach to different abiotic stresses. For example, the QTL effect itself may vary depending on the genetic background, environmental conditions, and plant growth stages. To offset these problems, only major QTLs of large effect should be pursued, as these are more likely to be stable and can justify the substantial investment required for fine-mapping and MABC.

New Marker-Assisted Breeding Strategies

Recent strategies have proposed selection approaches based on increasing the frequency of desirable alleles for larger numbers of QTLs in a population, for example up to 30 loci using marker-assisted recurrent selection (MARS; Bernardo, 2008). In MARS, multiple cycles of marker-assisted selection are

performed: F2 individuals with the desirable QTL alleles are crossed, and the progeny are screened with markers to undergo another round of selection, which continues until the desired frequency of favorable QTL alleles is achieved. In practice, the markers are used to calculate a genotypic value for each individual, and crosses are made based on this value to increase the frequency of favorable alleles in the population, which then reduces the size of the population required to identify an ideal genotype (Eathington et al. 2007). MARS schemes have proven popular with commercial breeding programs, and recently there has been more interest to use this strategy in the public sector as well, such as with the Molecular Breeding Platform proposed by the Generation Challenge Program and the Gates Foundation. Another strategy, referred to as genome-wide (or genomic) selection, proposes to use the combined effect on a trait from all the markers covering the genome instead of just using the significant loci as in MARS (Bernardo 2008). In this case, both phenotyping and genotyping the population is still needed to calculate the genotypic values of the progeny before selection, but information on specific QTLs is not necessary, since all markers are taken into account. Furthermore, efforts are being made to focus on predicting the performance of progeny from selected crosses even before phenotyping, which becomes possible once a large database of marker-trait associations is developed, as is currently being used in the Monsanto maize breeding program (Eathington et al. 2007). If this system will be employed in rice, it will require genome-wide marker scans to be performed on breeding lines that are then phenotyped in multi-environment field trials.

Sequencing More Rice Genomes

The completion of the high-quality DNA sequence of the rice genome by the International Rice Genome Sequencing Project was a landmark achievement. Although it took over eight years and was a massive undertaking, the fact remains

that it was a single rice genome, of the temperate *japonica* variety Nipponbare, that was sequenced out of the thousands of different rice genomes existing in the diverse germplasm from around the world. Nonetheless, there is tremendous value in having a "gold standard" reference sequence available, which will enable a host of downstream applications, not the least of which will be the re-sequencing of many more rice genomes in the years to come using the high-quality sequence of Nipponbare as a reference to align sequence fragments and provide added value to the 93-11 sequence data and other genomes to follow. At the same time, it will be important to have a *de novo* high quality *indica* and *aus* genome sequences as well, since there may be unique portions of the genome in those subgroups that are not present in Nipponbare, that would be hidden from any sequence assembly depending on the Nipponbare sequence, as was seen in a recent study on the *Pup1* QTL region which had several genes unique to Kasalath (Heuer et al. 2009). With cost-effective and rapid 2nd-generation sequencing techniques (such as Illumina Genome Analyzer IIx) whole genome sequencing of rice varieties will soon generate massive amounts of sequence data that will provide a valuable resource to the rice community.

SNP Genotyping Technologies

The successful implementation of these marker-assisted breeding strategies is dependent on having an efficient and robust genotyping system in place (Collard et al. 2008). For many years, simple sequence repeats (SSRs) have been the marker system of choice due to high polymorphism rates and the ability to run them on inexpensive gel electrophoresis equipment found in most labs. However, the routine integration of markers into modern breeding programs will require high-throughput genotyping platforms that can handle large numbers of samples at a low cost. Thus a new generation of markers based on single nucleotide polymorphisms (SNPs) is now rapidly overtaking SSRs due to new

SNP genotyping platforms that offer multiplexed sets of markers for different applications. SNP discovery efforts have begun to make large pools of SNPs available to the rice community (McNally et al. 2009; www.oryzasnp.org). Efforts are now being made to develop functional SNPs for foreground selection, to optimize 384 or 1536-plex SNP chips for low resolution genome scans, and to develop high resolution SNP chips with >40,000 SNPs for association genetics studies (personal communication, Susan McCouch, Cornell University). For example, an Illumina BeadXpress Reader at IRRI can genotype 384-SNPs on 96 samples with a two day protocol at less than $0.10/data point. As these genotyping tools are made more accessible to rice breeders, the full potential of marker-assisted breeding in mainstream breeding programs will finally be realized.

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Mapping of Brown Planthopper Resistance Gene Introgressed from *Oryza nivara* into Cultivated Rice, *O. sativa*

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Abstract

Brown planthopper (BPH) is one of the most destructive insect pests of rice in Asia. Wild rice species have been shown to be valuable sources for breeding resistant cultivars. In this study, SSR mapping of BPH resistance was conducted using an F_{10} family derived from a cross between an introgression line, '852T034', from *Oryza nivara* (Accession number 102165), and a susceptible *japonica* variety, 'Tainung 71'. Bulked analysis was used to evaluate the resistance of $F_{10:11}$ families. A BPH resistant gene contributed by 852T034 was mapped between two flanking SSR markers, RM16655 and RM3317, on the short arm of chromosome 4. This is the first time a BPH resistance gene derived from the wild species *O. nivara* was mapped. The tightly linked SSR markers will be facilitated marker-assisted breeding and provide the basis for map-based cloning of BPH resistant gene.

Key words: Brown planthopper, *Oryza nivara*, Wild rice, Genetic mapping, Marker-assisted breeding

INTRODUCTION

The brown planthopper (BPH), *Nilaparvata lugens* (Stal.), is one of the most destructive phloem-sap-sucking insect pests of rice (*O. sativa* L.) and can cause significant yield losses. Heavy infestations cause complete drying and plant death known as hopper burn. Not only the BPH feeds on the rice plant directly but also transmits rice grassy stunt virus and ragged stunt virus that cause severe disease (Khush and Brar, 1991). The application of BPH resistance genes has been recognized as the most economic, effective measure for BPH management and the most environmental friendly. Several resistance loci associated with BPH resistance have been reported and reviewed. Among these, *Bph1*, *bph2*, *Bph3* and *bph4* have been used extensively in rice breeding programs (Chen et al. 2006). In recent years, rice cultivars carrying *Bph1* or *bph2*, however, have lost their ability against BPH in Taiwan and the situation is getting worst probably related to global climate change.

Wild species of *Oryza* are potential source of new genes for resistance to BPH. Since few useful genes from wild germplasm accessions have been explored, there is still great potential for exploring novel genes (Xiao et al. 1998). In previous studies, eleven out of 21 BPH resistance loci derived from wild species have been reported in rice (Jena et al. 2006; Rahman et al. 2009). They are *Bph10* on the short arm of chromosome 12 from *O. australiensis* (Ishii et al. 1994), *Bph11(t)* and *bph12(t)* on the long arm of chromosome 3 and chromosome 4 from *O. officinalis* (Hirabayashi et al. 1998), *Bph12(t)* on the short arm of chromosome 4 from *O. latifolia* (Yang et al. 2002), *Bph13(t)* on the long arm of chromosome 2 from *O. eichingeri* (Liu et al. 2001), *Bph20(t)* and *Bph21(t)* on the short arm of chromosome 4 and the long arm of chromosome 12 from *O. minuta* (Rahman et al. 2009). Numerous studies have identified QTLs associated with BPH resistance on the adjacent chromosome regions near *Bph1* and *bph2* (Sharma et al. 2004). At least, six of these studies identified a major BPH resistance QTL located on the

short arm of chromosome 4 using both inter- and intra-species cross (Hunag et al. 2001; Yang et al. 2002, 2004; Huang et al. 2001; Sun et al. 2005; Rahman et al. 2009). In interspecific crosses, it was noticed that the wild accessions were the one always contributing the resistant allele at BPH resistance locus. a

Oryza nivara is an annual wild rice species and is possibly one of the direct ancestor of *O. sativa*. An introgression line '852T034' derived from a cross between *O. nivara* and *japonica* type Tainung 67 was selected and tested its resistance to BPH. The objectives of this study were to identify SSR markers closely linked to the BPH genes in order to be facilitated marker-assisted selection (MAS) in Taiwan rice breeding program and to map the major resistance genes on rice chromosome for further gene cloning.

Materials and Methods

Plant materials

The introgression line, 852T034, derived from an interspecific cross between Tainung 67 and a wild species *O. nivara* (Acc. No. 102165) show consistent resistance to BPH. 852T034 and Tainung 71 (a mega variety susceptible to BPH) were used as parents to develop a F_{10} population consisting 199 progeny for genetic analysis. The $F_{10:11}$ from each F_{10} plants were bioassay for BPH resistance. To test SSR markers linked to BPH resistance, a F_2 population derived form a cross between 852T034 and 871948, an *O. officinalis* susceptible introgression line, was generated for genetic analysis and $F_{2:3}$ families for BPH bioassay.

BPH bioassay

A pure BPH population was developed and maintained from single colony of BPH biotype 1 in the greenhouse facility of Chiayi Agricultrual Experiment Station of TARI. The bioassay was performed with bulk analysis and seedbox screening technique. Seedlings at three-leaf stage were infested with second- and

third-instar nymphs density of 2-3 nymphs per seedling. The reaction against the BPH was scored following the guidelines of Standard Evaluation System for Rice (IRRI, 2002). Taichung Native 1 susceptible to all biotype of BPH was used as the susceptible check and Mudgo (*Bph1*) and H105 (*bph2*) were used as the resistant checks.

DNA preparation and SSR analysis

Plant DNA was extracted from the leaves of rice plants using Wizard Genomic DNA extraction kit (Promega, United State). SSR analysis was performed according to the procedure of McCouch et al. (1997) with minor modification. The original source and motifs for all SSR markers used in this study could be found in the Gramene database (http://www.gramene.org/), McCouch et al. (2002) and IRGSP (2005). Amplification reactions were carried out in a 20 μl containing 50 ng DNA, 50 μM of each primer, 10x PCR buffer [100 mM Tris (pH 8.3), 500 mM KCl, 15 mM $MgCl_2$], 250 μM of each dNTP and 0.6 U of *Taq* polymerase (Protech Inc., Taiwan). PCR was performed in a PTC-200 Thermocycler (MJ Research Inc., United State) programmed as 5 min at 94°C, 35 cycles of 1 min at 94°C, 1 min at 55°C and 1.5 min at 72°C, with a final extension of 5 min at 72°C. Amplified PCR products were detected by electrophoresis in 3% SFR agarose gels (Amersco Inc., United State) staining with ethidium bromide.

Mapping QTL for BPH resistance

Linkage groups and the order of markers were determined using MAPMAKER/EXP 3.0 (Lander et al. 1987). The Kosambi mapping function was selected to correct the recombination frequency to genetic distances (cM). Marker regression and interval QTL mapping were carried out using Map Manager QTX 0.30 (Manly et al. 2001) with a likelihood ratio statistic (LRS) threshold 20 and a probability level of 0.01 for declaring the presence of putative QTLs.

RESULTS

BPH resistance evaluation

The BPH resistance phenotypes of the $F_{10:11}$ were evaluated when the 90% seedlings of the susceptible check 'TN1' were killed by BPH after infestation. The results showed the average severity scores of the parents '852T034' and 'Tainung 71' were 3.7 and 7.7, respectively. '852T034' showed the highest of BPH resistance. The severity scores of the 199 $F_{10:11}$ infested with BPH displayed a continuous distribution, ranging from 3 to 9, with two apparent peaks around 3 and 7 (Fig. 1).

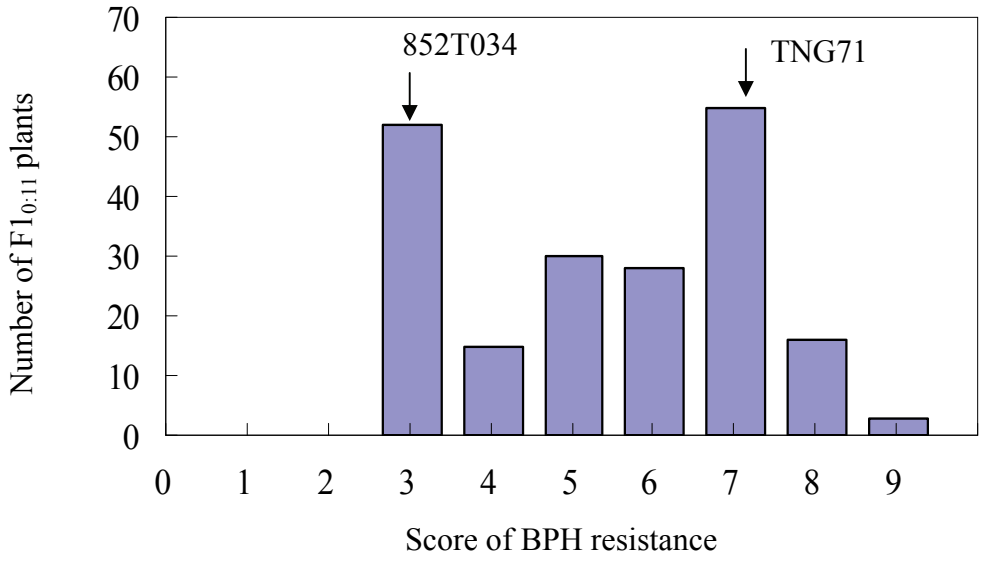

Fig 1. The frequency distribution of BPH resistance scores of $F_{10:11}$ lines derived from 852T034 × TNG71 in 2009 first crop**.**

Identification of SSR markers linked with the BPH resistance gene

To identify SSR markers tightly linked to BPH resistance, a total of 157 SSR markers, selected from 12 chromosomes with an interval of ~15 cM were tested. A linkage map was constructed by SSR genotyping 199 F_{10} individuals, and a major QTL for BPH resistance was identified. The BPH resistance locus detected with a LRS 136 was located between RM16655 and RM3317 on the short arm of

chromosome 4 (Fig. 2) and explained 50% of the phenotypic variance of BPH resistance in this population and the resistance was contributed by '852T034'.

Line	BPH	Chromosome 4						
		RM3536	RM16554	RM16563	RM16655	RM3317	RM7113	RM3308
TNG71	7.7	A	A	A	A	A	A	A
852T034	3.7	B	B	B	B	B	B	B
R006	4.3	B	B	B	B	B	B	B
R008	9.0	A	A	A	A	A	B	B
R010	7.7	H	A	A	A	A	B	A
R011	3.7	A	B	B	B	B	A	A
R012	9.0	A	A	A	A	A	B	H
R019	8.3	H	A	A	A	A	A	A
R022	5.0	B	B	B	B	B	A	B
R029	7.7	H	A	A	A	A	B	B
R032	3.0	B	B	B	B	B	B	A
R033	6.3	H	A	A	A	A	B	B
R036	6.3	A	A	A	A	A	B	B
R047	7.0	A	A	A	A	A	A	A
R074	5.7	H	H	A	H	H	B	B
R099	5.0	B	B	B	B	B	A	B
R118	4.3	B	B	B	B	B	A	A

Fig 2. Phenotype (BPH resistance) and graphical genotype of randomly selected F_{10} individuals. A: Tainung 71 allele, B: 852T034 allele, H: heterozygous

MAS test for the BPH resistance gene linked to SSR markers

The SSR markers RM16655 and RM3317 were further validated as R- or S-associated DNA markers by genotyping the F_2 progenies with different genetic background of Tainung 71. One hundred and forty-two F_2 seedlings from a cross between 852T034 and a susceptible *O. officinalis* introgression line 871948 were genotyped and $F_{2:3}$ families were used for BPH bioassay. 71% and 79% homozygous allele of 852T034 at RM16655 and RM3317 loci in the F2

population showed resistance scores ≤5 , respectively. More than 90% F_2 individuals detecting homozygous allele of 852T034 showed different degrees of increasing BPH resistance (Table 1). The results indicated linked SSR markers have great potential used in marker-assisted selection.

Table. Distribution of BPH resistance scores by genotypes of markers RM16655 and RM3317 in F_3 of 852T034 x 871948

Marker	Genotype[z]	Number of lines in BPH resistance score class						Total	Mean score
		RS<=5	(%)	5<RS<7	(%)	RS>=7	(%)		
RM16655	1/1	14	26	14	26	26	48	54	6.74
	1/2	15	50	7	23	8	27	30	5.57
	2/2	41	71	13	22	4	7	58	4.72
RM3317	1/1	14	25	16	28	27	47	57	6.74
	1/2	23	53	12	28	8	19	43	5.35
	2/2	33	79	6	14	3	7	42	4.55

[a] 1/1 denotes the homozygous genotype of '871948',1/2 denotes the genotype of the heterozygous, 2/2 denotes the homozygous genotype of '852T034'; RS, Resistance Score.

DISCUSSION

BPH is a major biotic stress in rice production in most Asian countries. In Taiwan, BPH resistance genes such as *Bph1* and *bph2* were introduced into *japonica* cultivars by conventional breeding methods. However, because of changes in BPH biotype and infestation patterns, varieties with the *Bph1* gene for resistance have become susceptible. It is also reported that biotype changes occurred because of the immigration of new biotypes of BPH from China, Vietnam and Philippines by summer wind since BPH never overwinters in Taiwan (Cheng and Huang, 2004). Therefore, identification of a new source of BPH resistance genes followed by introducing into *japonica* cultivars is an important objective in breeding programs.

Wild rice species are important resources of disease and insect resistance in crop genetic improvement (Tanksley and McCouch, 1996). Several wild species, including *O. latifolia*, *O. minuta*, *O. nivara*, *O. officinalis* and *O. punctata*, possess resistance to various biotypes of BPH (Renganayaki et al. 2002; Yang et al, 2004). In this study, a BPH resistance gene explained 50% phenotypic variations in the F2 population was mapped on the short arm of chromosome 4 and the resistance was contributed by '852T034' allele derived from *O. nivara* . This location is very close to BPH loci of *Qbp2*, *Bph12(t)*, *Bph15*, *Bph17* and *Bph20(t)* (Hunag et al. 2001; Yang et al. 2002, 2004; Huang et al. 2001; Sun et al. 2005; Rahman et al. 2009). It is very interesting to notice that BPH resistance genes derived from different wild rice species were mapped on the same region of chromosome 4. Disease resistance genes of plants were reported often clustering in the same chromosome region. It remains to be investigated whether the distribution of insect pests resistance genes is similar to that of disease resistance genes.

BPH populations can quickly overcome single resistance gene under natural conditions. New resistance genes are always needed for rice improvement and breeding against BPH. Therefore, the resistance genes of '852T034' can be a useful BPH resistance donor for a new resource of BPH resistance. The transfer of BPH resistance genes from wild species to different variety backgrounds can be greatly facilitated by using MAS (Tanksley and Nelson. 1996; Moncada et al. 2001). There has been great progress in the development of MAS for BPH in recent years. SSR and CAPS markers linked to *Bph1*, *bph2* and *Bph3* were used widely in Japan, Korean and Thailand MAS programs (Sharma et al. 2004; Cha et al. 2008; Jairin et al. 2009). But relatively few varieties or lines have been reported to be successfully developed by this method. One of the problems lies in the crossovers between markers and genes caused the segregation of the resistance in the population and deviated selection.

In this study, we conducted SSR mapping of wild species *O. nivara* and identified the SSR markers closely linked to BPH resistance locus, and verified by the

efficiency of MAS. Both RM16655 and RM3317 showed over 90% accuracy predicting BPH resistance in the F_2 generation derived from 852T034 x 871948. Thus RM16655 and RM3317 could be applied to the MAS of the trait of BPH resistance in rice breeding programs.

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Integration of genomics into breeding in rice

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ABSTRACT

Elucidation of the association between nucleotide and phenotypic changes has been a big challenge in plant molecular genetics. Toward this goal, we have been involved in the genetic dissection of natural phenotypic variations in rice and have identified several genes involved in complex traits, including heading date, shattering habit, pre-harvest sprouting, root morphology, disease resistance and eating quality. To enhance the power of genetic dissection of complex phenotypes, we are developing several mapping populations, such as recombinant inbred lines and chromosome segment substitution lines, which will be required to extract the useful alleles from natural variants. Marker assisted selection has been applied for the development of heading date and durable resistance. To facilitate allele mining using novel plant materials, we have also embarked on the genome-wide discovery of single nucleotide polymorphisms (SNPs). In particular, to overcome a shortage of SNPs among temperate japonica cultivars, we have attempted whole-genome sequencing of several cultivars using next-generation sequencing approaches. Although Japanese cultivars are closely related genetically, about 67,000 SNPs have been discovered between Nipponbare (reference) and Koshihikari by this approach. This SNP discovery has led to the development of an array-based SNP genotyping system in Japanese rice cultivars. Large-scale

genotyping of these SNPs has made it possible to visualize pedigree haplotypes for the Japanese landraces and modern cultivars. These new efforts in genomics have opened up new opportunities to accelerate not only the genetic dissection of complex traits, but also the improvement of rice cultivars.
Key words: Quantitative trait locus, Genetic mapping, Marker assisted selection, Single nucleotide polymorphism

INTRODUCTION

It is about 4 years since the whole rice genome was decoded (IRGSP 2005). The sequence information has provided new tools for genetics and has created a new paradigm of selection strategy in plant breeding. Many phenotypic traits of economic interest are controlled by multiple genes and often show complex and quantitative inheritance. Recent progress in rice genomics has had a great impact on the genetic dissection of such traits into single genetic factors or quantitative trait loci (QTLs) (Yamamoto *et al*., 2009). These resources have already contributed to both our understanding of biological phenomena in plants, and to the application of genomics tools to the development of new crop cultivars. Genes with agronomic value have been tagged by DNA markers and have been introduced into elite cultivars by marker-assisted selection (MAS). Technological innovations in large-scale sequencing and genotyping have opened new possibilities in rice genetics and breeding. This paper introduces our recent activity on the genetic dissection of complex traits, marker-assisted introgression, and pyramiding of agronomic traits, and future prospects in genomics-assisted breeding of rice.

Uncovering the Naturally Occurring Variations in Complex Traits

In the last decade, much effort has been paid to the genetic and molecular dissection of complex traits. An excellent example is the analysis of heading date in rice. Heading date is a key determinant for the adaptation of rice to different

cultivation areas and cropping seasons. Therefore, control of heading date is a leading objective in rice breeding. Many genetic studies have been conducted for QTL mapping of heading date using advanced backcross progeny (Yano *et al*., 2001). Fifteen QTLs, called *Heading date* (*Hd*)*1–Hd3a* and *Hd3b–Hd14*, have been detected by using several kinds of progeny from a cross between *japonica* cultivar 'Nipponbare' and *indica* cultivar Kasalath (Yano *et al*., 2001). Among them, nine QTLs—*Hd1*, *Hd2*, *Hd3a*, *Hd3b*, *Hd4*, *Hd5*, *Hd6*, *Hd8*, and *Hd9*—were mapped as single Mendelian factors (Yano *et al*., 2001; Lin *et al*., 2000; 2003). Detection of QTLs for heading date has allowed further genetic analyses, such as the development of nearly isogenic lines (NILs), analysis of epistatic interactions among QTLs, and map-based cloning. *Hd1* has been found to encode a protein with zinc finger and CCT motifs and to be an ortholog of *Arabidopsis CONSTANS* (Yano *et al*., 2000). *Hd6* and *Hd3a* were found to encode a casein kinase 2 alpha and an *Arabidopsis* FT-like protein (Takahashi *et al*., 2001; Kojima *et al*., 2002). A major QTL, *Early heading date 1* (*Ehd1*), for heading date was detected on chromosome 10 by using a BC_1F_1 population derived from a cross between cultivar T65 and an accession of another cultivated species, *Oryza glaberrima* (Doi *et al*., 1998). Further analysis revealed that *Ehd1* encodes a B-type response regulator (Doi *et al*., 2004). In all cases of QTL cloning for heading date, large-scale linkage mapping was required to narrow the candidate genomic region for the QTLs. These efforts led us to identify functional nucleotide polymorphisms in *Hd1*, *Hd6*, and *Ehd1*. To comprehensively dissect the genetic factors controlling naturally occurring variations in rice flowering, we have performed a QTL analysis using 12 populations derived from crosses of the *japonica* cultivar Koshihikari with cultivars and lines that originate from various regions in Asia. By QTL mapping, several QTLs were detected on the 12 rice chromosomes, some of which were shared among the different cross combinations. The chromosomal locations of these QTLs corresponded to those detected in Nipponbare and Kasalath (Yano *et al*., 2008). However, the allelic

effects of each QTL varied among the parental combinations used, suggesting that a large proportion of the wide range of phenotypic variations in flowering time could be generated by the combination of different alleles of the corresponding QTLs. These genetic and molecular studies have definitely contributed to our understanding of heading date in rice (Izawa, 2007; Tsuji *et al*., 2008).

Much effort has also been paid to the analysis of other complex traits, such as grain size, shattering habit, disease resistance, and environmental stress tolerance. These activities are summarized in the "Gramene-QTL" database (Jaiswal *et al*. 2002). Around 8000 QTLs have been detected so far. Among them, several with major effects have already been cloned by a map-based strategy (Yamamoto *et al*., 2009).

Current Status of Marker-assisted Selection in Rice Breeding

Once genes controlling traits with economic and agricultural interest are cloned or mapped precisely on the respective chromosomes, it becomes possible to use information such as gene sequences and chromosomal locations in breeding programs. Since the paradigm of MAS emerged nearly 20 years ago, much effort has been invested in the practice of MAS. Several examples of the development of NILs with particular traits in elite cultivars have already been reported. Submergence by deep water causes severe stress to rice in Southeast Asia, where flooding occurs during the monsoon season. A major QTL, *Submergence 1* (*Sub1*), was detected near the centromere of chromosome 9 (Xu *et al*., 2000). The underlying gene was cloned (Xu *et al*., 2006), and the *Sub1A* allele was introgressed by MAS into an elite cultivar grown widely in Asia. The resultant lines showed promising performance in yield and other agronomic traits, as well as tolerance to submergence (Neeraja *et al*., 2007; Septiningsih *et al*., 2009). Four QTLs for rice heading date—*Hd6*, *Hd1*, *Hd4*, and *Hd5*—were introgressed from Kasalath into Koshihikari by MAS to enhance the cropping potential of Koshihikari, one of the leading cultivars in Japan (Takeuchi *et al*., 2006). As a

result, NILs of Koshihikari with early and late heading dates have been successfully developed. The size of the introgressed chromosomal segments in those lines was very small: 300 to 600 kb in NILs for *Hd1*, *Hd6*, and *Hd5*. Precise information on the chromosomal locations of the genes allowed breeders to minimize the length of the substituted chromosome segments containing the target QTLs. That study clearly demonstrated the potential power of MAS in rice breeding. Recently, cloning of a durable blast resistance gene, pi21, has been cloned and this achievement led us to solve a linkage drag of pi21, associating with high level of blast resistance and inferior eating quality. Clarification of precise location of pi21 allowed us to develop several markers, which could be used for the dissection between blast resistance and eating quality (Fukuoka e*t al*. 2009). MAS also offers a new concept in breeding: Once NILs with particular economic value are developed, gene pyramiding can be performed by simple crossing between them (Ashikari and Matsuoka, 2006). To develop a new line with lodging resistance and high yield, the combination of two genes controlling semi-dwarfing and grain number were successfully introduced into Koshihikari (Ashikari *et al*., 2005). This concept can also be applied to multiple genes controlling specific traits. Four QTLs controlling partial resistance to rice blast in upland rice have been successfully pyramided into lowland rice cultivars by MAS (Fukuoka and Saka, 2006; Saka *et al*., 2007).

Allele Mining for Rice Breeding

Many successes have been achieved in cloning and MAS of particular QTLs in rice. However, these successes have largely depended on allelic differences. In most cases, the allelic difference was relatively large, allowing reliable determination of the chromosomal location. One major QTL, *Grain number 1a*, was successfully isolated by a map-based strategy (Ashikari *et al*., 2005). This finding contributed both to our understanding of the genetic control of spikelet development in rice, and to MAS to improve grain number per panicle. However,

in general, one major QTL alone is not enough to acquire the level of phenotypic performance needed. To this end, it would be necessary to combine genes with major and minor effects. Owing to the statistical power of detection, it is usually very difficult to detect QTL with minor effects in QTL analysis using F_2 and recombinant inbred lines (RILs). To solve this problem, we developed chromosome segment substitution lines (CSSLs) (Ebitani *et al*., 2005; Takai *et al*., 2007; Ando *et al*., 2008). In these novel plant materials, a particular chromosomal segment from a donor line is substituted into the genetic background of the recurrent line. The substituted segments cover all chromosomes in a whole set of lines. The potential of CSSLs in QTL detection has been demonstrated in many ways. We have established a systematic research flow for exploration and cloning of useful genes (Fig. 1). For example, CSSLs can be used in genetic analysis to associate QTLs with particular chromosomal regions and to quickly develop NILs of target regions containing QTLs of interest. In general, when an association is detected between a chromosomal region and a trait, it is often difficult to validate the QTLs, especially those with very small genetic effects. In such a case, NILs are required in order to analyze genetic effects in detail (Miura *et al*., 2001; Sato *et al*., 2003; Ueda *et al*., 2004). Because CSSLs normally have one chromosomal region substituted, they can be used as NILs themselves or as starting material to develop NILs. Such NILs enable us to combine two or three QTLs in one genetic background in order to clarify epistatic interactions among them (Lin *et al*., 2000; 2003; Yamamoto *et al*., 2000). Furthermore, once we detect significant differences between the CSSLs and the recurrent parental line, comparison of the size of the substituted segments enables us to delineate candidate chromosomal regions of QTLs (substitution mapping). If a significant difference is found between a particular CSSL and the recurrent parent, a large mapping population can be easily produced by a simple crossing of the CSSL with the recurrent parent. Map-based cloning of the QTLs detected can be started quickly by using such plant materials. A simple survey of target traits on the CSSLs allows us to detect

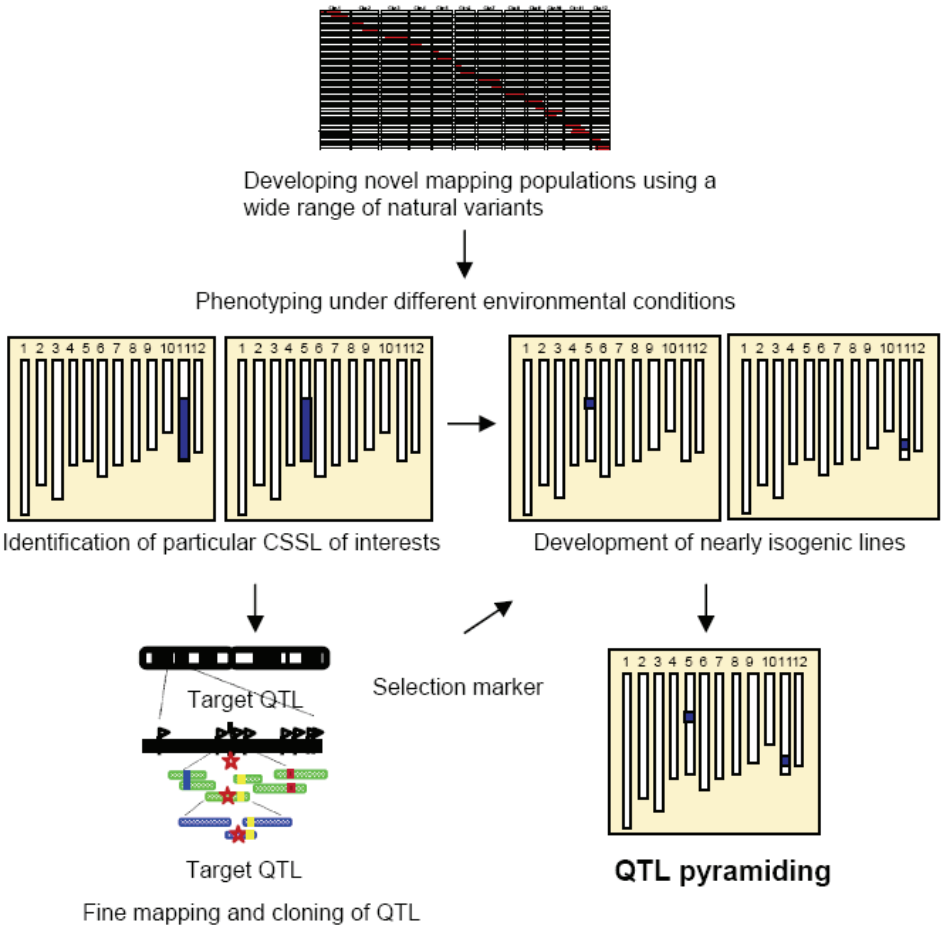
Developing novel mapping populations using a wide range of natural variants
Phenotyping under different environmental conditions
Identification of particular CSSL of interests
Development of nearly isogenic lines
Target QTL
Selection marker
Target QTL
QTL pyramiding
Fine mapping and cloning of QTL

Fig.1 Systematic research flow for the exploration and utilization of natural variations in rice

minor phenotypic differences with reference to the recurrent parent, because there is almost no effect of background genetic noise (i.e., allelic effect of other QTLs). Although the resolution of QTLs in terms of linkage mapping and the power to detect epistatic interactions are not comparable with those given by primary mapping populations such F_2s and RILs, the use of CSSLs can facilitate the discovery of valuable alleles from donor chromosome segments. To enhance the potential of CSSLs, we are now developing CSSLs from a wide range of cross combinations, with Koshihikari as the recurrent parent. Donor parental lines are *indica* and *japonica* cultivars, including some of our core collections (Kojima *et al*., 2005).

Genome-wide Discovery of SNPs and Development of Genotyping Arrays

The genetic dissection of natural variations requires novel and effective genotyping and phenotyping. New technology recently enabled us to perform massive analysis of sequences (Blow, 2007; Hutchison, 2007). This method, called next-generation sequencing technology, has opened new opportunities for polymorphism discovery (Lister *et al*., 2009). In Arabidopsis, this system has been applied to two natural accessions to perform genome-wide single-nucleotide polymorphism (SNP) and insertion/deletion (InDel) discovery (Ossowski *et al*., 2008). This method may provide a unique opportunity to identify nucleotide polymorphisms between genetically closely related strains, such as Japanese cultivars. In addition, a system of simultaneous genotyping of thousands of SNPs on the genome scale has been recently developed (Steemers and Gunderson, 2007). The combination of both new technologies has also facilitated the genetic dissection of complex traits by QTL mapping and whole-genome association studies (Nordborg and Weigel, 2008).

We have detected about 5000 SNPs from our core collection of Asian cultivated rice accessions (Ebana *et al*., 2007). In addition, we have sequenced the whole genome of Koshihikari by using a next-generation sequencing technology, and

have discovered a comprehensive set of SNPs and enhanced the potential power of allele mining among *japonica* cultivars (Nagasaki *et al*., 2008). An enormous amount of genomic sequences, equivalent to more than 20 genomes, has been obtained for Koshihikari, and a consensus sequence of Koshihikari established from the mapped sequences covers nearly 80% of the reference Nipponbare sequences. Comparison of these two sequences has provided more than 60 000 SNPs. These candidate SNPs were validated by an array-based SNP detection system. These SNPs will facilitate the genetic analysis of traits with economic interest among temperate *japonica* cultivars.

From Gene Selection to Genome Selection

The development of a genome-wide SNP typing system allows us to perform large-scale genotyping of recent breeds of rice. We have genotyped a large set of SNPs using landraces, old cultivars, and current leading cultivars (Yamamoto *et al*., 2008). By including their pedigrees, it has been possible to visualize the genotype of the whole genome (pedigree haplotype) of those successions. This analysis provided valuable information on current rice breeding. For example, we can visualize the proportion of the genome derived from Koshihikari, and which chromosomal regions are shared among current leading cultivars. In addition, recombination events that contributed to the improvement of cultivars can also be identified. Previously, MAS has targeted particular chromosomal regions or genes, because DNA markers are genotyped one by one. However, the simultaneous analysis of a large number of SNPs provides a new concept of selection criteria in breeding: It might be possible to perform selection based on a whole-genome genotype image and haplotype images of particular chromosomal regions.

Future Prospects

MAS, developed as an armchair theory about 20 years ago, has now been realized by the accumulation of information on precise chromosomal locations and tightly

linked DNA markers flanking genes with major effects. However, its application to rice breeding has been limited in the precision mapping of genes with minor effects and their introduction into elite cultivars. This limitation is not matter of genomics—the tools and information—but depends on phenotyping methods and plant materials; for example, it depends on how we discover useful genes from diverse germplasm and how we establish reliable phenotyping methods. Integration of all resources, such as tools, analytical methods, and plant materials, will be necessary to facilitate further dissection of complex traits with agricultural importance, such as yield performance, drought tolerance, and grain quality. These efforts will make genomic-assisted breeding more effectively and practically.

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Genetic engineering of "C_4 rice": Rationale and performance of transgenic rice plants expressing cyanobacterium and maize CO_2 concentrating mechanism genes

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Rice is the staple food for half of the world's population, and 90% of the world's rice is grown and consumed in Asia. Thus, rice production is important to the food security of the world. The world's population is projected by FAO to rise from the current 6.5 billion to 9 billion in the next 40-50 years, with most of the population increases occurring in rice-consuming countries. Thus, rice yield must be increased by at least 50% over the next 40 years to prevent malnutrition for the 700 million Asian that currently rely on rice for more than 60% of their daily calorific intake. After the successful application of the hybrid rice technology in recent years, rice yields have been substantially improved and the wide adoption of the technology will further increase the global rice production. However, hybrid rice technology alone can not meet the demands in the long run. Furthermore, traditional breeding approach is time-consuming and ineffective in solving the food shortage problems. In contrast, molecular breeding, a technology combining genetic engineering and traditional breeding, will be a powerful and effective approach to overcome the food production problems. Theoretical analysis has suggested that the yield increases required to match the projected

population growth can only be achieved by increasing the efficiency with which photosynthesis uses solar energy. Our interest is to increase rice growth and productivity by placing the C_4 pathway of photosynthesis into rice.

Rationale for genetic engineering of "C4 rice"

Photosynthesis is the most important metabolic process relative to plant growth and crop productivity because carbohydrates account for more than 85% of the dry weight in plants. The C_3 pathway of photosynthesis evolved first in an environment enriched in CO_2. However, over geologic time atmospheric CO_2 levels decreased and consequently plants evolved several CO_2 concentrating mechanisms (CCMs) in response to decreases in atmospheric CO_2 level: bicarbonate transport system in cyanobacteria, algae and aquatic plants and the C_4 pathway and Crassulacean acid metabolism (CAM) in terrestrial plants (Price *et al.*, 2008). Under low CO_2 or alkaline water conditions, cyanobacteria use high affinity bicarbonate transporters (e.g., inorganic carbon transporter – ictB or sodium bicarbonate transporter – sbtA) to pump in bicarbonate as a major carbon source. This adaptive CCM allows cyanobacteria to survive unfavorable growth conditions. Higher vascular plants have two main photosynthetic mechanisms, namely the C_3 and C_4 pathways. The most productive crops, such as corn, sorghum and sugarcane, use the C_4 pathway while most of the important agronomic crops, such as rice, wheat and potatoes, use the C_3 pathway. The photosynthetic efficiency is much higher in C_4 than in C_3 plants, due to the C_4 CO_2 concentrating mechanism, which suppresses the photorespiratory activity associated with Rubisco. Therefore, C_4 plants are more productive and use CO_2 (**Fig. 1**), minerals (**Fig. 2**) and water with a higher efficiency (250-350 g H_20 used/g dry matter produced as versus 550-850 g H_2O used/g dry matter produced for C_3 plants), especially under high light, warm temperature (**Fig. 3**) and drought environments. As compared to the C_4 crop corn, rice is a C_3 crop and has a lower photosynthetic efficiency and thus a lower yield.

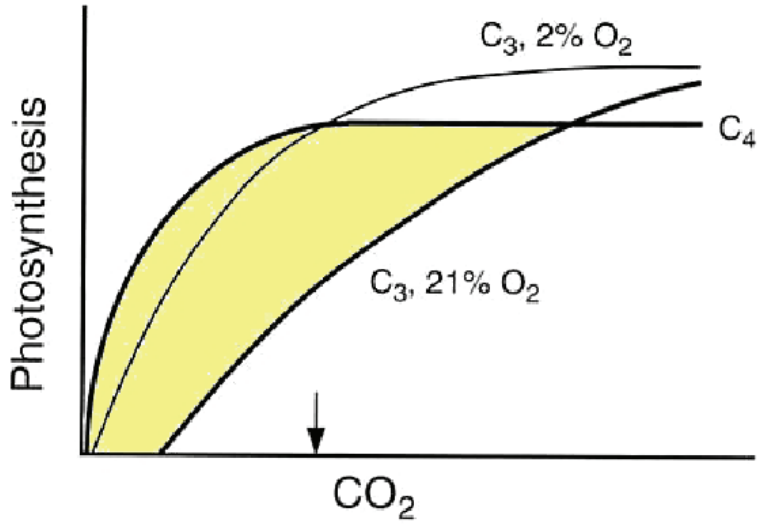

Fig. 1. Photosynthetic CO_2 responses of C_3 and C_4 photosynthesis at 2 and 21% O_2 (adopted from Ku *et al.,* 1996).

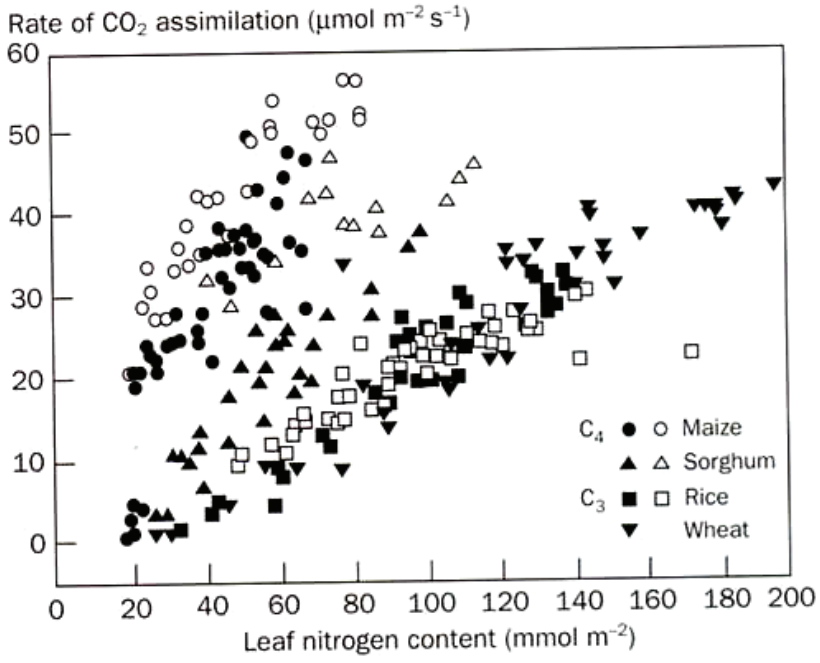

Fig. 2. Photosynthetic nitrogen responses of two C_3 plants (rice, wheat) and two C_4 plants (maize, sorghum) (adopted from Sage, 2004).

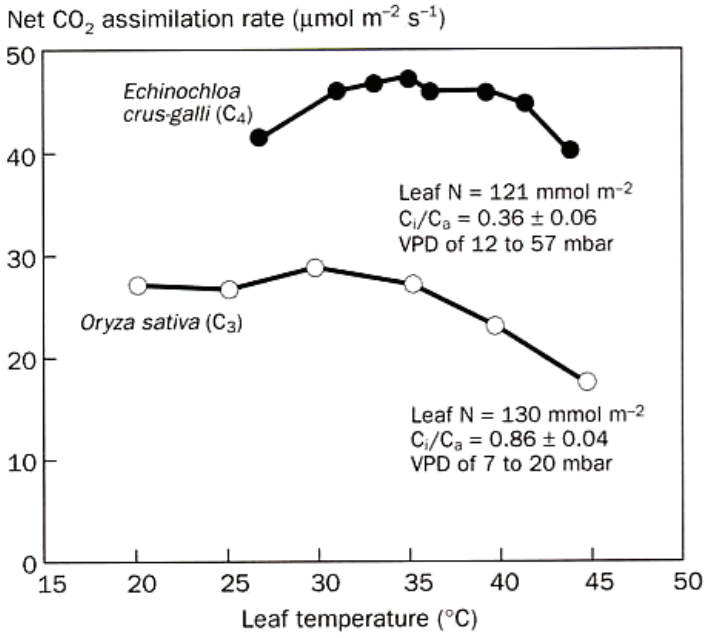

Fig. 3. Photosynthetic temperature responses of rice (C_3) and *Echinocloa crus-galli* (C_4) (adopted from Sage, 2004).

The evolution of C_4 plants from C_3 plants occurred at least 45 times in angiosperms, but the changes are complex. Most C_4 plants compartmentalize photosynthetic reactions between two morphologically distinct cell types that are arranged in concentric circles around the vascular veins, so called Kranz leaf anatomy (**Fig. 4**). This anatomical change coupled with the C_4 pathway of photosynthesis endow C_4 plants a superior photosynthetic capability. There is no doubt that it is extremely ambitious to generate C_4 rice. However, the polyphyletic evolution of C_4 pathway suggests that the transition from C_3 to C_4 is relatively simple and provides cause for optimism. Genes encoding all the enzymes involved in C_4 pathway are present in C_3 plants, although their expression levels are much lower than in C_4 plants. Also, some plants, such as *Eleocharis*, exhibit plasticity in switching from C_3 to C_4 under certain environmental conditions. With a better understanding of the formation of Kranz leaf anatomy and gene regulation in C_4 plants, it may be possible to generate C_4 rice with enhanced photosynthesis, growth and yield.

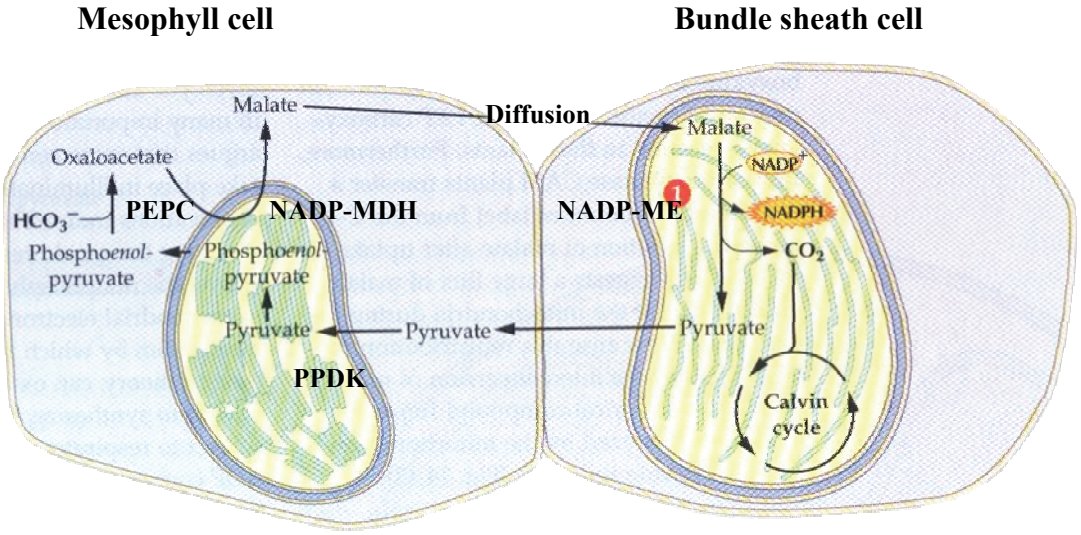

Fig. 4. The C_4 pathway in NADP-malic enzyme (ME) type C_4 plants, such as maize, sugarcane and sorghum. The key enzymes of the pathway are phosphoenolyruvate carboxylase (PEPC), NADP-malate dehydrogenase (NADP-MDH), NADP-malic enzyme (NADP-ME), and pyruvate orthophosphate dikinase (PPDK) (adopted from Bucanon *et al.*, 2001).

Strategies for creating C4 rice

Since rice is a C_3 plant, which lacks Kranz leaf anatomy, it will be rather difficult to introduce the C_4 pathway of maize (**Fig. 4**) in the single mesophyll cells of rice. However, the C_3 aquatic plant *Hydrilla verticillata* is capable of inducing a primitive C_4 pathway in the mesophyll cells when grown under low CO_2 conditions (**Fig. 5**). This primitive C_4 pathway in Kranz-less leaf is apparently effective in reducing photorespiration. It is also clear that this primitive C_4 pathway is very similar to that of maize, but have all the reactions taking place in a single mesophyll cell. The earlier strategy on engineering C_4 rice has been concentrating on mimicking this system in rice. Transgenic rice lines have been produced from Kitaake (japonica) to overexpress several maize C_4 photosynthesis genes independently or simultaneously: phosphoenolpyruvate carboxylase (PEPC, Ku *et al.*, 1999)), pyruvate Pi dikinase (PPDK, Fukayama *et al.*, 2001),

NADP-malic enzyme (ME, Takeuchi *et al.*, 2001), CK (PEPC x PPDK) and CKM (PEPC x PPDK x ME). In general, the photosynthetic efficiency and grain yield of these transgenic plants are higher than that of untransformed rice plants (Ku *et al.*, 2000; Ku *et al.*, 2001, Jiao *et al.*, 2001), but high level expression of ME causes photooxidation in transgenic plants due to metabolic imbalance (Takeuchi *et al.*, 2000; Tsuchida *et al.*, 2001). This is due to the competition between PSII and NADP-ME for NADP in the mesophyll chloroplast of ME transgenic rice plants. Depletion of NADP pool in the chloroplast by high activities of NADP-ME renders electron being accepted by oxygen molecule for production of harmful oxyradicals. NADP-ME type C_4 plants avoid this problem by modifying its bundle sheath chloroplast without having PSII activity in the non-stacked thylakoid membrane (**Fig. 1**) whereas the aquatic plant *H. verticillata* may avoid this problem by living in a relatively low light condition. Thus, a functional C_4 CO_2 concentrating is still lacking in these transgenic rice plants.

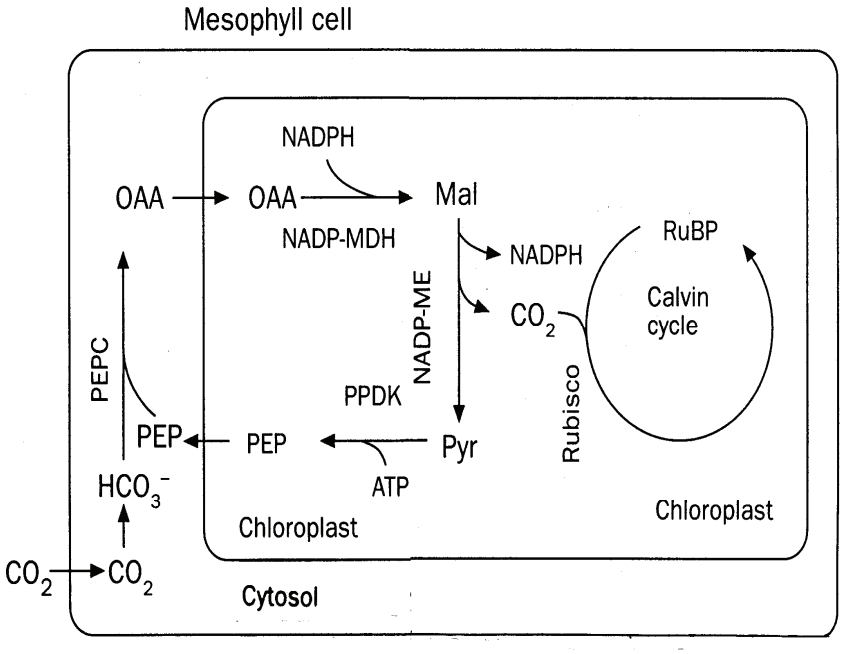

Fig. 5. The primitive C_4 pathway in *Hydrilla verticillata*, an aquatic plant (Magnin *et al.*, 1997)

To avoid metabolic imbalance in engineering C_4 rice by mimicking the C_4 pathway of *Hydrilla* or maize, our recent efforts have been concentrated on creating a synthetic CO_2 concentrating mechanism in rice by combining the CCMs from both cyanobacterium and maize. In this simplified scheme (**Fig. 6**), inorganic carbon transporter from cyanobacteria is expressed on plasmamembrane of mesophyll cells to pump in bicarbonate, followed by carboxylation of bicarbonate in the cytosol by maize PEPC to form oxaloacetate (OAA) and subsequent decaroxylation of OAA by maize PEP carboxykinase in the chloroplast (PCK). With this synthetic pathway, bicarbonate, the substrate for PEPC, is directly transported into the cell and OAA formed is decarboxylated by PCK upon entering the chloroplast without the need to be reduced to malate with

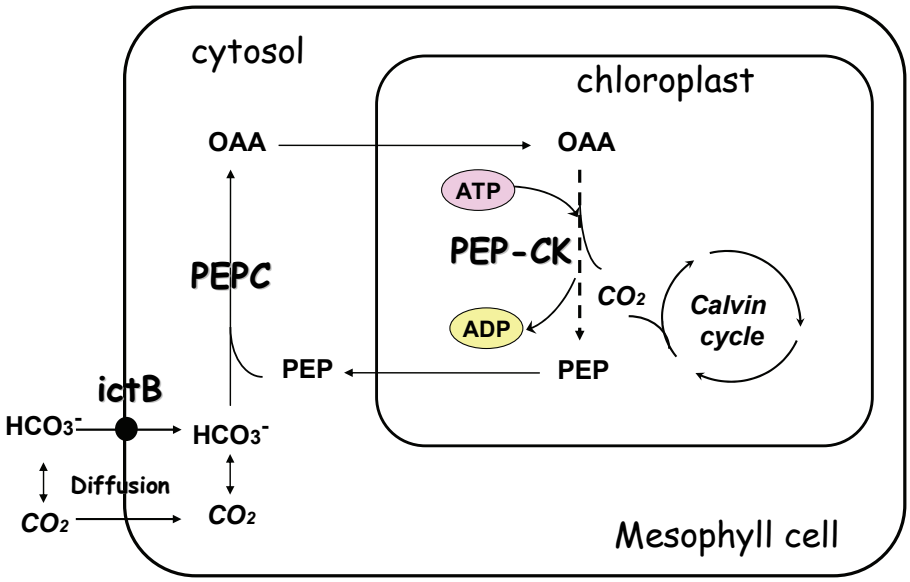

Fig. 6. Creation of a functional C_4 pathway in the C_3 mesopyll cells. The three key steps are: (1) transport of biocarbonate across membrane by inorganic carbon transporter (ictB or sbtA), (2) carboxylation by phosphoenolpyruvate carboxylase (PEPC) in the cytosol, and (3) decarboxylation of oxaloacetate (OAA) in the chloroplast by PEP carboxykinase (PEP-CK).

the input of NADPH energy. Furthermore, the decaoxylation of OAA to release CO_2 and PEP by PCK requires only one ATP and PEP is directly used for PEP carboxylation whereas in the NADP-ME type C_4 pathway it requires two ATP to regenerate PEP from pyruvate after decarboxylation of malate by ME. Thus, energetically this synthetic CO_2 concentrating mechanism will be more favorable. So far, we have produced transgenic rice from Tainoung 67 (japonica subtype) to constitutively express (1) ictB or sbtA gene from cyanobacteria, (2) intact maize PEPC gene and (3) maize PCK gene.

(1) ictB transgenic rice: All transgenic rice lines expressing the cyanobacterium ictB transporter exhibited enhanced activities of Rubisco (25-30%) and PEPC (10-20%), higher carboxylation efficiency (15-20%), higher photosynthesis rates (10-30%), and lower photosynthetic CO_2 compensation points (5-10%). Consistently, the transgenic rice plants produced more panicles per plant (10-120%) and more grains (10-70%), relative to untransformed plants. The enhancements in growth and grain yield are closely related with the increased photosynthetic capacity among the transgenic lines. This study demonstrates that the simple CCM from cyanobacterium can largely improve the photosynthetic efficiency, growth and productivity of C_3 crops, such as rice.

(2) PEPC transgenic rice: Transgenic rice lines that expressed high levels of the maize PEPC with PEPC activities up to three fold higher than that of maize have been obtained. Compared with untransformed wild type rice plants, PEPC transgenic plants showed higher photosynthetic rates (10-20%), especially under high light conditions, higher light and CO_2 use efficiencies, and higher grain yields in the field (up to 50-60%).

(3) PCK transgenic rice: PCK is a cytosolic enzyme. In order to target the enzyme to the chloroplast for effective decarboxylation of OAA in transgenic rice plants, the maize gene is linked to the chloroplast targeting

sequence from tomatao RbcL in the transformation vector. Compared to untransformed rice plants, PCK transgenic plants showed higher total biomass (5-20%), heavier grains (11-16%) and increased grain yield (3-12%). Most importantly, feeding OAA to leaf enhanced the photosynthetic rates of transgenic plants, indicating it is functional in the chloroplast by supplying more CO_2 in the chloroplasts. An earlier study by Suzuki *et al.* (2001) also produced transgenic rice overexpressing PCK gene isolated from PCK subtype C_4 plant *Urochloa panicoides*.

A functional C_4 pathway requires the coordination of several biochemical steps located in different cellular and intracellular compartments. Traditional cross hybridization was used to generate transgenic plants simultaneously expressing these enzymes in the same plants. Homozygous transgenic plants expressing both PEP and ictB or both PEPC and PCK have been obtained for further characterization. Preliminary evaluation showed that these hybrid transgenic rice plants have significantly higher biomass and grain yields. It is anticipated that simultaneous expression of ictB, PEPC and PCK in rice is likely to confer a functional C_4 photosynthesis, leading to increased photosynthesis and productivity. In addition, we have also evaluated the potential of transferring these CCM genes to hybrid rice by crossing the various transgenic lines with elite rice cultivar, such as 93-11, to combine heterosis and C_4 photosynthetic traits. Preliminary observation suggests this is a feasible approach.

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Development of Single Nucleotide Polymorphism (SNP) Markers and Applications in Rice Breeding

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Single nucleotide polymorphisms (SNPs) are attractive because they are abundant, genetically stable, and amenable to high-throughput analysis. Using the genome sequence information of eight rice cultivars (*Oryza sativa* L. ssp. *japonica* and ssp. *indica*) and a wild rice accession (*Oryza rufipogon* Griffith), we were able to identify 7805 polymorphic loci including SNPs and insertions/deletions (InDels). The frequency of SNPs in the rice genome varied between different chromosomes. To illustrate the usefulness of SNPs as a marker system, we have successfully generated 331 SNP markers (Fig.1) between an *indica* cultivar Guang-Lu-Ai 4 (GLA4) and a wild rice accession (*Oryza rufipogon* Griffith, W1943). These SNP markers were used in the so-called "genome breeding" method to incorporate highly desirable agronomic traits such as semidwarfness and variations in heading date to the japonica cultivar Koshihikari, one of the most popular rice varieties in Japan.

Although Koshihikari is grown in many regions throughout the country, it is highly susceptible to lodging. In order to improve the lodging-susceptibility, we developed a breeding strategy to incorporate the semidwarfness trait into the Koshihikari genome. The *indica* cultivar IR24 was used as donor of the semidwarf-1 (*Sd-1*) gene. It was crossed with Koshihikari (Fig. 2a, b), and the resulting F_1 plants were backcrossed with Koshihikari, producing BC_1F_1 plants. All BC_1F_1 plants were analyzed by using 108 SNPs distributed throughout the genome (Fig. 2c). The SNP markers were successfully used to assess the graphical genotypes of all progenies at the seedling stage. The substitution rate with chromosomes of the recurrent parent theoretically showed a normal distribution. This graphical approach facilitated the selection of plants with a

heterozygous *sd-1* and a maximum amount of Koshihikari genome for use in the next backcross. Subsequent backcrossings and selections were performed similarly, and in the BC_4F_2 generation, an individual with Koshihikari chromosomes except in the *sd-1* region was obtained (Fig. 2f). This plant shows the morphological phenotypes of Koshihikari except for semidwarfness. Breeding was completed in only 24 months from the first cross. Although the semidwarf Koshihikari has about 4 Mb of IR24-derived genetic materials, it is possible to further narrow down the substituted region to a single open reading frame by screening a large number of selfed population with SNP markers very close to *sd-1*. This new type of semidwarf Koshihikari is now widely cultivated in several prefectures in central Japan.

We have also used the SNP markers in developing six isogenic lines (ILs) of Koshihikari with different heading dates. A similar approach involving successive backcrossing was adapted. In this case, the *indica* cultivar GLA4 was used as donor of heading date QTLs. GLA4 was crossed with Koshihikari, and the resulting F_1 plants were backcrossed to Koshihikari. Further successive backcrosses to Koshihikari were carried out for 2 times, and a total of 175 SNP markers were used to analyze the backcross progenies of each generation. In the BC_3F_2 generation, six individual plants with Koshihikari chromosomes except for the target region of a heading date QTL were selected (Fig. 3). These ILs can be the candidate of new types of Koshikari varieties for practical breeding of suitable heading date highly adapted to different rice cultivating regions in Japan.

Our results demonstrate the immense potential of SNPs as molecular markers in rice breeding. Any identified genes for useful traits can be objects of “genome breeding”. Genetically distant parents that have been considered unsuitable for breeding because of their inferior traits can be used, and are even preferable because SNP markers can be developed much more easily between genetically distant parents. It is also a very practical strategy because the number of plants that must be analyzed per generation can be very small, and only one plant is needed to create the next backcross. Thus, even a limited space in a greenhouse is sufficient for multiple breeding projects.

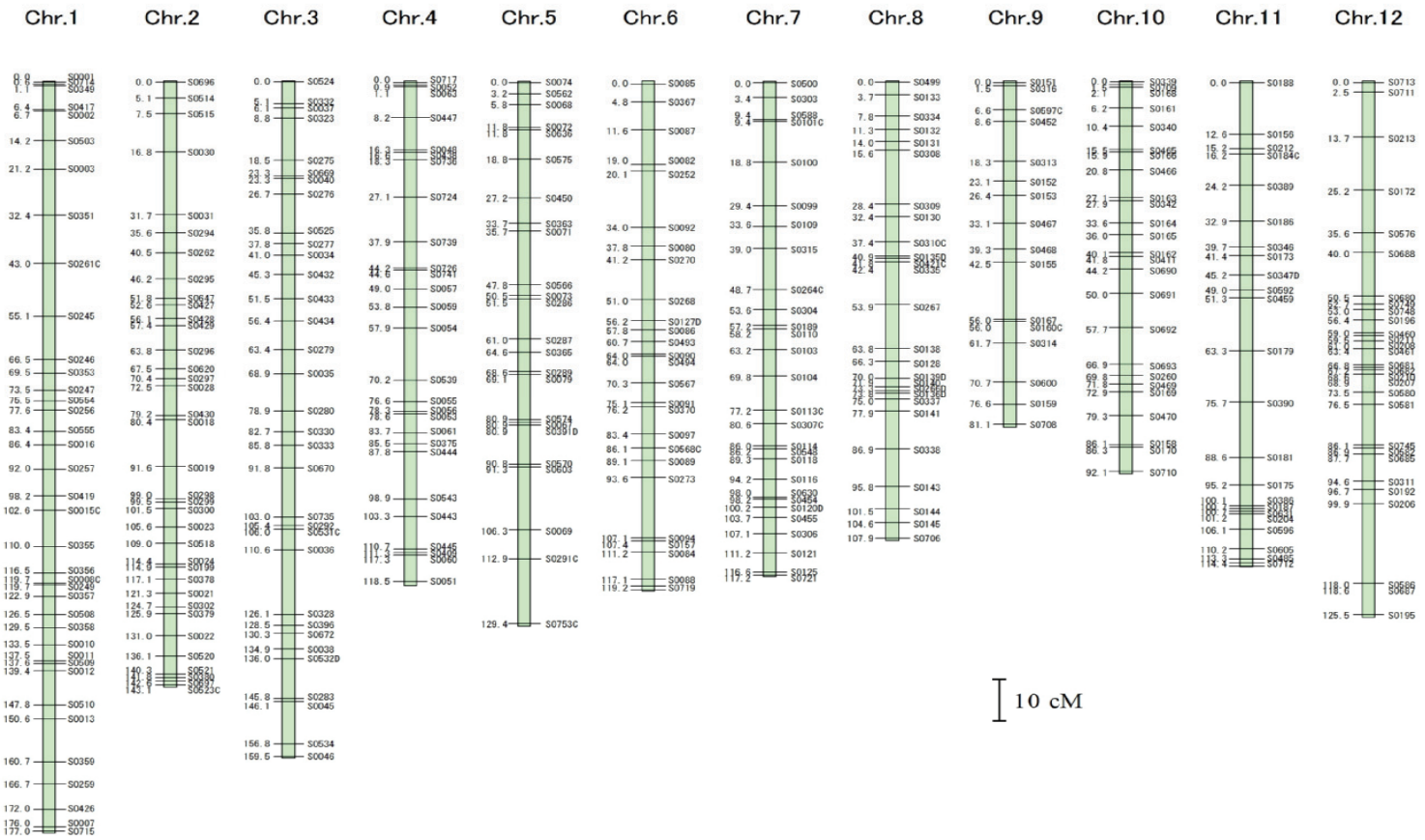

Fig. 1. A genetic map of 331 SNP markers.

The linkage analysis was carried out using 184 F_2 individuals derived from a cross between an *indica* cultivar GLA 4 and a wild rice accession W1943. Map distances expressed in centimorgans (cM) were calculated using the Kosambi function.

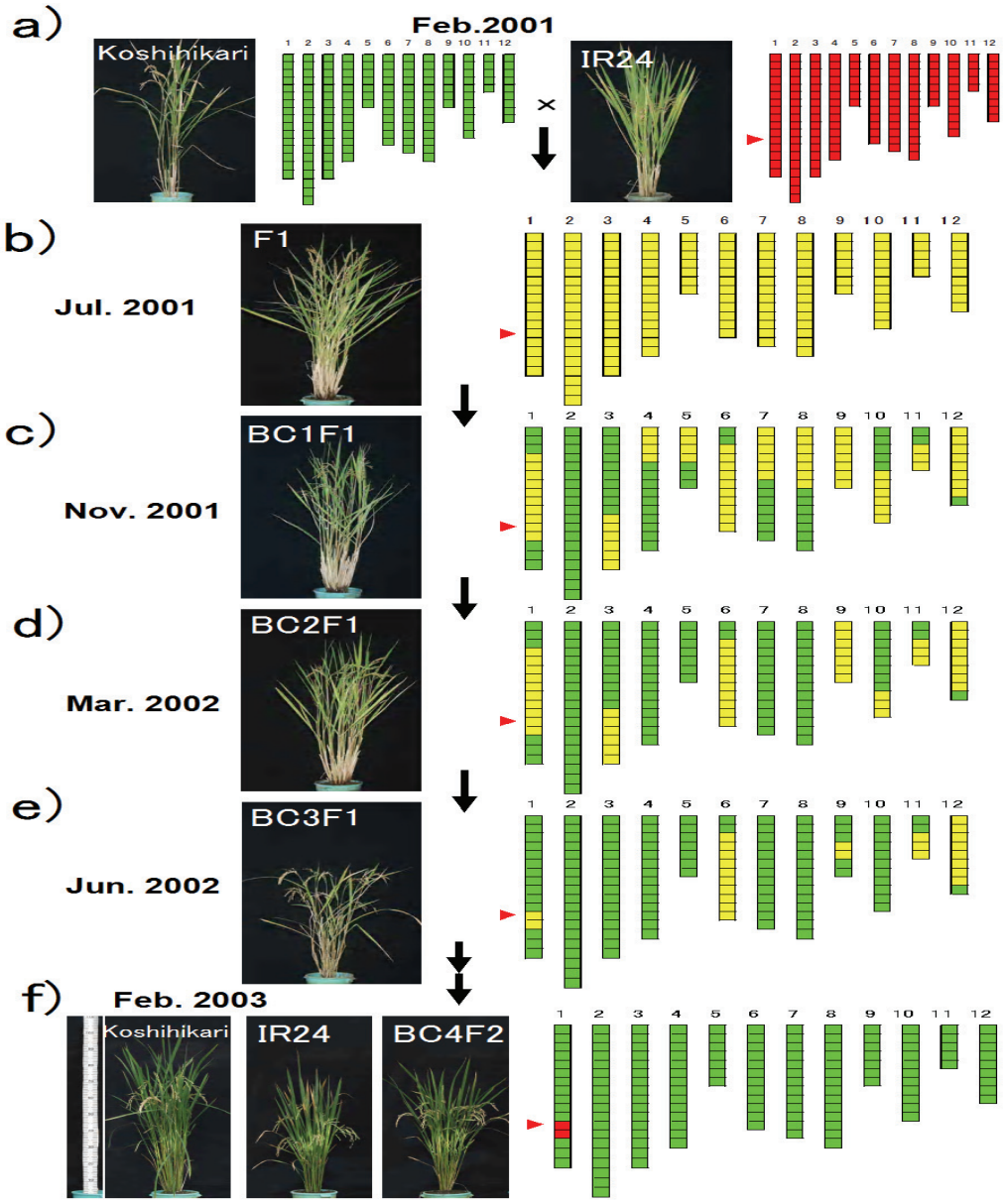

Fig. 2. Procedure for genome breeding of semidwarf Koshihikari.

The rice semidwarf-1 (sd-1) gene (position shown by red arrowhead) was introduced from *indica* cultivar IR24 (a, right) into the leading Japanese cultivar Koshihikari (a, left). Figures to the right of the photos show the graphical genotype of the selected plant at each backcross generation, drawn from 108 SNPs distributed throughout the genome. Koshihikari and IR24 homozygous regions are shown in green and red, respectively, and heterozygous regions are shown in yellow.

Fig. 3. Koshihikari and six isogenic lines (ILs) with variations in heading date.

Days-to-heading (DTH) of Koshihikari was 100 days. DTHs of Wasei 1, Wasei 2, Wasei 3, Bansei 1, Bansei 2, and Bansei 3 were 92 days, 87 days, 81 days, 104 days, 105 days, and 110days, respectively. DTH was measured as the number of days from sowing to heading date in Tsukuba, Japan in 2008.

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UPDATE FOR ELITE JAPONICA VARIETY

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ABSTRACT

A new concept called Variety Update for rice molecular breeding was introduced. We updated four traits of *japonica* elite variety Koshihikari and bred four new varieties of Koshihikari in this work. The idea of Variety Update was from computer software and digital informatics. To improve on less desirable traits of elite variety only through replacing a small chromosomal fragment, was our concept.

Key words: Variety Update, SNP marker, Rice breeding, QTL, Trait

INTRODUCTION

The origin of agriculture led to the domestication of many plant species and to the exploitation of natural resources. It took almost 10,000 years for food grain production to reach 1 billion tons, in 1960, and only 40 years to reach 2 billion tons, in 2000 (Khush, 2001). This unprecedented increase, which has been named the "Green Revolution," resulted from improved crop varieties, combined with the application of agronomic practices. It was known that the semi-dwarf gene *sd1* played the main role in the green revolution. Gene *sd1* produces strong rice culm, with the result that a rice plant has lodging tolerance and is a high-yield type. Since the Green Revolution, many rice varieties with *sd1* have been bred and used in rice production in various Asian countries to increase food supply. China, to counter this dearth, built up its hybrid rice-breeding system with the *sd1* gene to develop higher-yield varieties.

However, rice varieties in Japan have shown no change, even in the Green Revolution. Bred 50 years ago, Koshihikari is still the most popular rice variety even now. It has taken over about 38% of rice paddy areas in Japan even at present; while other paddies have been generally inadaptable for the cultivation of Koshihikari. This indicates it has been the optimum rice variety for the last five decades in Japan. Koshihikari is considered to be of very good eating quality, but has lower yields. This fact indicates that the breeding technology of the Green Revolution only played a role in increasing the yield of rice variety, but could not improve the eating quality with *sd1* gene only.

The goal of key technology in crop breeding is to make certain progress in new varieties. Looking at the huge numbers of rice varieties bred every year, however, it appears that farmers (users) were not shown what traits were improved in the new varieties. Rice farmers in Japan would certainly never use the old-variety Koshihikari if an improved Koshihikari existed - just as a computer user would never depend on a ten-year-old laptop.

Since the two agriculturally important subspecies of rice, called *indica* and *japonica,* were published (IRGSP, 2005, Han and Zhang, 2008), DNA markers used in genetic analysis, QTL finding, and map-based cloning are easily made. In rice breeding, DNA marker selection methods were established and used in the actual breeding. In this paper, we show a new concept that is different from DNA marker selection methods or MAS (Marker-Assisted Selection), called "Variety Update."

MATERIALS AND METHODS

The idea of Variety Update is from computer software, the internet, iPhone and others that improve on old versions. This concept involved two steps: one, to find out any bugs, undesirable traits, defects and so on; and the other, to solve such problems, and to add new functions, thereby achieving progress. Koshihikari is the preferred rice variety in Japan but it has some defects such as weak lodging tolerance, lower yield, low regeneration ability, strong photosensitivity and others. Here, we attempted a procedure we call "Variety Update" to improve the less desirable traits of Koshihikari.

Updating the Lodging Tolerance of Koshihikari

We identified the position of culm length QTL in the semi-dwarf gene *sd1* region in chromosome 1. The QTL showed that the chromosome fragment of *indica* variety HABATAKI in the *sd1* region has the effect of reducing Koshihikari culm length by some 15 cm. We decided to use the chromosome fragment to update the lodging tolerance of Koshihikari. Semi-dwarf gene *sd1* was isolated (Sasaki et al., 2002), and we bred the introgression line ILA with Koshihikari background, which has the chromosomal fragment of HABATAKI's *sd1* region. We crossed ILA and Koshihikari to make the F1 plant and made the F2 segregation population ILAF2 from the seeds of the F1 plant. We selected a plant ILAF2-1, which is heterozygous in the *sd1* locus and homozygous Koshihikari type in the vicinity of *sd1* locus. Then the seeds of plant ILAF2-1 were used to make the second segregation population ILAF3. A plant ILAF3-1 was selected from the segregation population of ILAF3. ILAF3-1 is heterozygous in the *sd1* locus but homozygous Koshihikari type in both sides of *sd1*.

Finally, an F4 plant ILAF4-1 was selected from a segregation population ILAF4 derived from the seed of ILAF3-1. ILAF4-1 is homozygous HABATAKI type in the *sd1* locus and homozygous Koshihikari type in others. ILAF4-1 is the new Koshihikari variety, called Koshihikari Eichi 4, which is the result of this Variety Update for the lodging tolerance of Koshihikari.

Updating the Grain Yield of Koshihikari

We had the idea to increase grain yield of Koshihikari by making its panicle size larger. We found a QTL *Yq1* in chromosome 1. The allele of HABATAKI in the *Yq1* region has the effect of increasing the grain number of the main panicle. After that, we isolated the QTL as *Gn1a* gene (Ashikari et al., 2005). We bred the introgression line ILB with Koshihikari background, which has the chromosomal fragment of HABATAKI's *Gn1a* region. We crossed ILB and Koshihikari to obtain the F1 plant and made the F2 segregation population ILBF2 by growing the seeds of the F1. We selected a plant ILBF2-1, which is heterozygous in the *Gn1a* locus and homozygous Koshihikari type in the vicinity of *Gn1a* locus. Then the

seeds of plant ILBF2-1 were used to make the second segregation population ILBF3. A plant ILBF3-1 was selected from the segregation population of ILBF3. ILAF3-1 is heterozygous in the *Gn1a* locus, but homozygous Koshihikari type in both sides of *Gn1a*.

Lastly, an F4 plant ILBF4-1 was selected from a segregation population ILBF4, which was derived from the seeds of ILBF3-1. ILBF4-1 is homozygous HABATAKI type in the *Gn1a* locus and is homozygous Koshihikari type in others. ILBF4-1 is the new Koshihikari variety called Koshihikari Eichi 2, being the result of Variety Update in the grain yield of Koshihikari.

Updating the Callus Regeneration Ability of Koshihikari

Koshihikari is one of the most important *japonica* varieties used for food production, such as the *indica* variety IR64. However, Koshihikari has only very low regeneration ability of callus. For future gene function and genome function research, we decided to update this trait. We found a QTL *PSR1* in chromosome 1. The allele of *indica* variety Kasalath has the effect of increasing the ability of callus regeneration of Koshihikari. We isolated the QTL as *PSR1* gene after that (Nishimura et al., 2005). We bred the introgression line ILC with Koshihikari background, which has the chromosomal fragment of Kasalath's *PSR1* region; crossed ILC and Koshihikari to obtain the F1 plant; and made the F2 segregation population ILCF2. We selected a plant ILCF2-1 which is heterozygous in the *PSR1* locus and homozygous Koshihikari type in the vicinity of *PSR1* locus. Subsequently, the seeds of plant ILCF2-1 were used to make second-segregation population ILCF3. A plant ILCF3-1 was selected from the segregation population of ILCF3. ILCF3-1 is heterozygous in the *PSR1* locus, but homozygous Koshihikari type in both sides of *PSR1*.

Finally, an F4 plant ILCF4-1 was selected from a segregation population ILCF4 derived from the seed of ILCF3-1. ILCF4-1 is homozygous Kasalath type in the *PSR1* locus and homozygous Koshihikari type in others. ILCF4-1 is the new Koshihikari variety called Koshihikari Eichi 1, which is the result of this Variety Update for the callus regeneration ability of Koshihikari.

Changing the Photoperiod Sensitivity of Koshihikari

As mentioned, Koshihikari is the preferred variety in Japan, shown by about 38% of rice paddies being used for its rice production. However, Koshihikari could not be adapted to all areas in the country. The reason is that maturity will be too late if the variety is grown in northern Yamagata Prefecture (northern latitude of 38 degrees). And, its maturity will be too early if grown in southern Aichi Prefecture (southern latitude of 35 degrees). Therefore, we decided to change the photoperiod sensitivity of Koshihikari. A QTL gene *Hd1* is a known gene for photoperiod sensitivity in *japonica* varieties and had been isolated (Yano et al., 2000). We found a QTL *QTS1* in *Hd1* region of chromosome 6. The allele of indica variety HABATAKI in this region has the effect of changing the photoperiod sensitivity of Koshihikari. We bred the introgression line ILD with Koshihikari background, which has the chromosomal fragment of HABATAKI's *Hd1* region; and crossed ILD and Koshihikari to have the F1 plant and make the F2 segregation population ILDF2. We selected a plant ILDF2-1 from the segregation population ILDF2, which is heterozygous in the *Hd1* locus and homozygous Koshihikari type in the vicinity of *Hd1* locus. Next, the seeds of plant ILDF2-1 were used to make second-segregation population ILDF3. A plant ILDF3-1 was selected from the segregation population of ILDF3. ILDF3-1 is heterozygous in the *Hd1* locus, but homozygous Koshihikari type in both sides of *Hd1*.

Finally, an F4 plant ILDF4-1 was selected from a segregation population ILDF4, which was derived from the seeds of ILDF3-1. ILDF4-1 is homozygous HABATAKI type in the *Hd1* locus and is homozygous Koshihikari type in others. ILDF4-1 is the new Koshihikari variety called Koshihikari Eichi 3, which is the result of this Variety Update.

RESULTS

Results of Updating the Lodging Tolerance of Koshihikari

ILAF4-1 is homozygous HABATAKI type in the semi-dwarf gene *sd1* locus and homozygous Koshihikari type in others. The F5 seed from the ILAF4-1 was used to test the phenotype in the rice fields. The generation after that, derived from seeds of the ILAF4-1

plant, is called the new-variety Koshihikari Eichi 4. The culm length (stem length excluding panicle) of Koshihikari Eichi 4 was 82.9 cm whereas that of Koshihikari grown in Chiba Prefecture, Japan was 96.9 cm. The difference of plant height between Koshihikari Eichi 4 and Koshihikari was 14 cm, which is apparent in the rice field (Fig.1-A,B,C). Koshihikari Eichi 4 has a shorter culm length and better lodging tolerance than that of Koshihikari (Fig.1 D). However, there are no differences between Koshihikari and Koshihikari Eichi 4 excluding the above traits (Fig.1 F). We also measured the length of chromosomal fragment transferred from HABATAKI included *sd1* locus. The length of this fragment is from 90-840 kb.

Results of Updating the Grain Yield of Koshihikari

ILBF4-1 is homozygous HABATAKI type in the *Gn1a* locus and is homozygous Koshihikari type in others. The F5 seeds from the ILBF4-1 were used to test the phenotype in the rice fields. The generation after that from selfing plant ILBF4-1 is called Koshihikari Eichi 2 as a new variety. The grain number of main panicles of Koshihikari Eichi 2

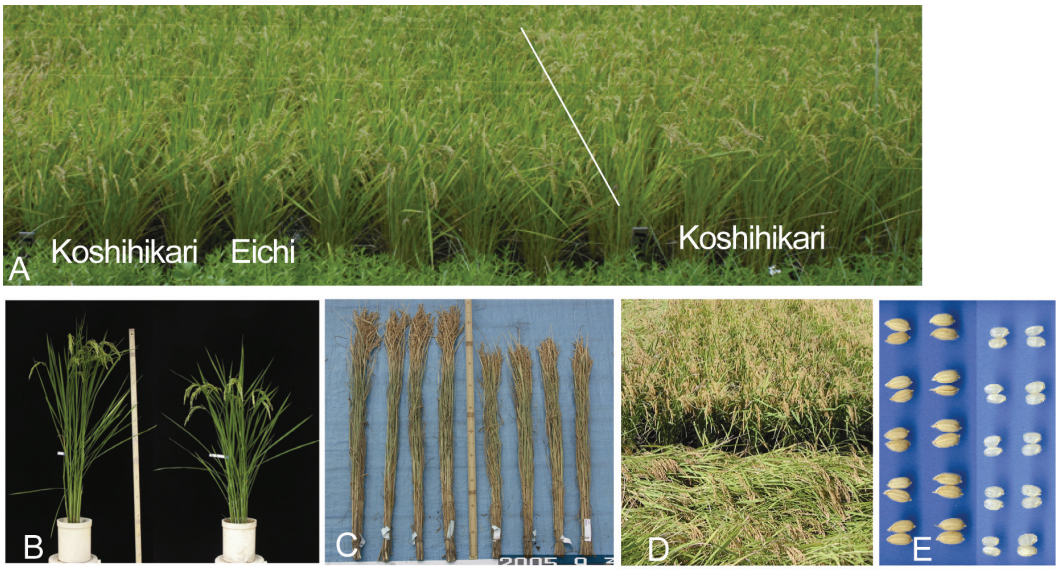

Fig.1. The differences between Koshihikari and Koshihikari Eichi 4. A: The plant height in rice field. B: Koshihikari Eichi 4 (right) and Koshihikari (left). C: The harvested plants, Koshihikari Eichi 4 (right) and Koshihikari (left). D: The lodging tolerance, Koshihikari Eichi 4 (upper), Koshihikari (lower). E: The seeds (left) and brown rice (right) of Koshihikari Eichi 4 (right) and Koshihikari (left).

averaged 196 when grown in Nagoya, Japan in 2006, whereas Koshihikari was 171 grains. There are also differences in panicle grain density between Koshihikari Eichi 2 (10.0 grains/cm) and Koshihikari (8.89 grains/cm) (Fig.2 C). However, there are no significant differences between Koshihikari and Koshihikari Eichi 2 excluding the above traits (Fig.2 A, B, D). We also measured the length of chromosomal fragment transferred from HABATAKI in the Koshihikari Eichi 2 included *Gn1a* locus. The length of this fragment is from 37kb-245 kb .

Results of Updating the Callus Regeneration Ability of Koshihikari

ILCF4-1 is homozygous Kasalath type in the *PSR1* locus and is homozygous Koshihikari type in others. The F5 seeds from the ILCF4-1 were used to test the phenotype in the rice fields. The generation after that from plant ILBF4-1 is the new variety called Koshihikari Eichi 1. The regeneration ability of Koshihikari Eichi 1 is higher than that of Koshihikari and similar to that of gene donor Kasalath (Fig. 3 C). However, there are no significant differences of the agricultural traits for Koshihikari and Koshihikari Eichi 1 excluding the above traits (Fig. 3 A, B).

Fig.2. The differences between Koshihikari and Koshihikari Eichi 2. A: The plant in rice field. B: The plant type, Koshihikari Eichi 2 (right) and Koshihikari (left). C: The panicles Koshihikari Eichi 2 (lower) and Koshihikari (upper). D: The seeds (left) and brown rice (right) of Koshihikari Eichi 2 (right) and Koshihikari (left).

Results of Changing the Photoperiod Sensitivity of Koshihikari

ILDF4-1 is homozygous HABATAKI type in the *Hd1* locus and homozygous Koshihikari type in others. The F5 seed from the ILDF4-1 was used to test the phenotype in the rice fields. The generation after that from plant ILDF4-1 is called Koshihikari Eichi 3 as the new variety. The heading date of Koshihikari Eichi 3 was July 27, 2006 when grown in Nagoya, Japan, whereas that of Koshihikari was August 7 of that year. The time from sowing to heading of Koshihikari Eichi 3 averaged 77 days whereas for Koshihikari, it was 88 (Fig.4 A, B). There are also different culm lengths for Koshihikari and Koshihikari Eichi 3, because of the different heading dates (Fig. 4 B). However, there are no significant differences between Koshihikari and Koshihikari Eichi 3 excluding the above traits.

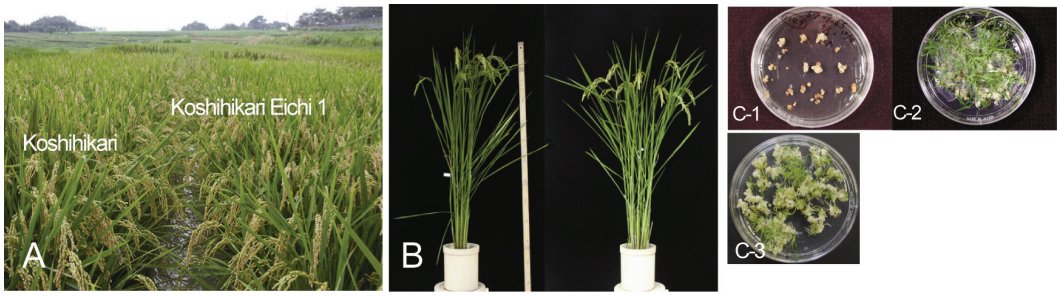

Fig.3. The differences between Koshihikari and Koshihikari Eichi 1. A: The plant in rice field. B: The plant type, Koshihikari Eichi 1 (right) and Koshihikari (left). C: The regeneration ability, Koshihikari (C-1), Kasalath (C-2), Koshihikari Eichi 1 (C-3).

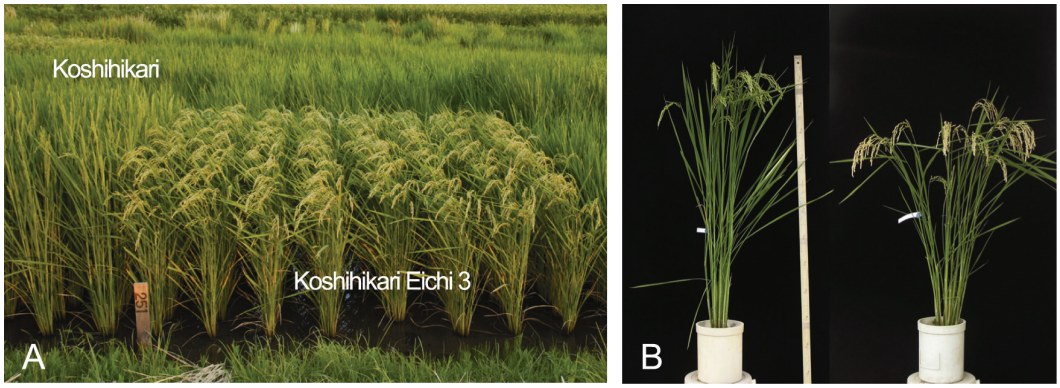

Fig.4. The differences between Koshihikari and Koshihikari Eichi 3. A: The plant in rice field, Koshihikari Eichi 3 headed earlier than Koshihikari. B: The plant type, Koshihikari Eichi 3 (right) and Koshihikari (left).

DISCUSSION

The aim of the work reported herein was to establish a new marker selection method call Variety Update, and to make certain progress in new varieties. The idea is from computer software or digital informatics (e.g. we attempt to update the *japonica* elite variety Koshihikari). Our goal was to improve Koshihikari through replacing one of its small chromosomal fragments with that of a donor's having a better gene (allele). It was most important in this concept to only modify target traits of Koshihikari. That meant we avoided as much as possible, changing other traits. Therefore we tried to keep the chromosomes of Koshihikari in the new-bred variety by replacing a very small chromosomal fragment of Koshihikari.

Deciding Which Trait(s) Should be Updated

The first step for our Variety Update was to find out any "bugs" in the elite variety. In other words, to decide which trait or traits should be modified. And next was to find the donor genome by QTL analysis. It was tempting to consider that many traits of Koshihikari should be modified, because it is a well-known and appreciated variety long used in rice production in Japan. We decided that the target traits for modification are: lodging tolerance, panicle size, callus regeneration ability, and photoperiod sensitivity. The traits of disease resistance and/or insect tolerance were also attractive targets for updating, also to improve the variety. However, it is necessary to discover less desirable traits of elite variety for updating by one's own knowledge and experiences in such methodology.

Selecting a Donor Genome

The important step in our method was to find a donor genome including a gene that has the desired effect of updating the targeted trait. Therefore we carried out the QTL analysis with the segregation population derived from Koshihikari and the donors selected. The results from the QTL analysis will show the effect of the donor genome in each detected QTL.

Therefore, the biggest QTL with the biggest effect should be selected for the update. However, one should retry the QTL analysis if the effect of the donor allele was ineffective.

Using Introgression Lines

Introgression lines were bred by backcross and DNA marker selection methods: cross the Koshihikari and the donor lines to obtain the F1 plant and then backcross with Koshihikari. The introgression line was selected that has only the donor chromosomal fragment in the target region. Other chromosomes and chromosomal fragments except the target region should be homozygous-type of Koshihikari.

We used 140 SNP markers to search all chromosomal regions and showed the introgression lines ILA, ILB, ILC and ILD do not have the donor chromosomal fragment except for the target regions. However, this is not to disavow the possibility that some donor chromosomal fragments exist still between the SNP markers. It is better to remove all such small donor chromosomal fragments in the IL genome by using more DNA markers and selecting markers distant equally.

Obtaining Gene Information of the Detected QTL Locations

To use only a very small chromosomal fragment to update less desirable traits of Koshihikari, one must know the given position of the gene in the chromosome. While the orthodox way is to identify it, information from published research papers will also help one identify gene position. Much information was available on this research; and the major genes *sd1, Gn1a, PSR1, Hd1* for these traits had been isolated (Sasaki et al., 2002, Nishimura et al., 2005, Ashikari et al., 2005, Yano et al., 2000). The locations in each region for these genes were the best references to identify each fragment of donor chromosome. In the final analysis one should identify a given position for each gene, with this methodology. It is also an effective way to discover a new gene, should your own results differ from those of others.

The Donor Chromosomal Fragment Size in the Updated Varieties

Our policy in this Variety Update method was to replace a minute DNA fragment of Koshihikari, to avoid changing other Koshihikari traits. Therefore, one needs genotype a multitude of plants from the segregation population. However, it would be expensive to genotype a large population. In our experiment we genotyped from 5,000 to 20,000 plants for each target trait. One point of genotype will cost USD1 so one target will cost $10,000 to $40,000 for genotyping if one genotypes two points for one plant.

It was not clear how big the donor DNA fragment should be for the replacement. Size in this method would depend on whether there are undesirable genes in the target region. It is no problem should the fragment be bigger if there is no undesirable gene there, and the cost will be less. Therefore, the size also depends on the relation between the donor genome and the elite variety Koshihikari. Even if that was unclear, we were trying to keep the size of the donor DNA fragment to less than 500kb for our Koshihikari Eichi series.

We updated four traits for Koshihikari and bred 4 new varieties called Koshihikari Eichi 1, Koshihikari Eichi 2, Koshihikari Eichi 3 and Koshihikari Eichi 4. These four varieties were applied for and allowed as the new Koshihikari varieties used in food production by the Japanese government. However, as said above, some small DNA fragment of donor may still exist between markers. Thus, these varieties also have to be under observation for a time.

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International Symposium on Rice Research in the Era of Global Warming

Program

Date/Time	Topic/Activity
6 Oct., 2009 (Tues.)	
08:30-09:30	Registration
09:30-09:50	Inaugural Session **Welcome Address** Dr. Dah-Jiang Liu, Director General of TARI 行政院農業委員會 農業試驗所 劉所長大江 **Opening Remarks** Dr. Yu-Tsai Huang, Deputy Minister of COA 行政院農業委員會 黃副主任委員有才 Dr. Hong-Pang Wu, Professor of Academia Sinica 中央研究院 鄔教授宏潘
09:50-10:00	Group Photo
10:00-10:20	Tea/Coffee Break
Session 1. Effect of Global Warming on Rice Production	
	Moderator: Dr. Hong-Pang Wu (鄔宏潘教授) **Institute of Plant and Microbial Biology, Academia Sinica, Taiwan**
10:20-11:05	Effect of Global Warming on Rice Production and Mitigating Strategies 全球暖化對稻作生產之影響及調整策略 Dr. Motohiko Kondo (National Institute of Crop Science, National Agriculture and Food Research Organization, Japan)
11:05-11:50	Changes in Water Distribution during Rice Grain-Filling in Relation to Formation of Milky-White Type of Grain Chalkiness by High-Temperature Stress 高溫逆境下對水稻穀粒充實與白堊質之影響 Dr. Tsutomu Ishimaru (National Institute of Crop Science, National Agriculture and Food Research Organization, Japan)
11:50-13:30	Lunch
Session 2. Rice Breeding Strategy (I)	
	Moderator: Dr. Chang-Sheng Wang (王強生副教授) **Department of Agronomy, National Chung Hsing University, Taiwan**

13:30-14:15	To Promote Rice Grain Nutrition by Marker-Assisted Breeding 以分子輔助育種提昇稻米營養價值 Dr. Yann-Rong Lin (林彥蓉博士) (Department of Agronomy, National Taiwan University, Taiwan)
14:15-15:00	Deployment of Genetic Variation and Marker-Assisted Breeding for Biotic Stress Tolerance in Rice 水稻遺傳變異與分子標誌輔助育種在生物逆境抗性之有效利用 Dr. CasianaVera Cruz (International Rice Research Institute, Philippines)
15:00-15:30	Tea/Coffee Break

Session 3. Rice Breeding Strategy (II)

Moderator: Dr. Min-Tze Wu (吳明哲組長)
Biotechnology Division, Taiwan Agricultural Research Institute, Taiwan

15:30-16:15	Marker-Assisted Breeding for Abiotic Stress Tolerance in Rice－Progress and Future Perspectives 水稻非生物逆境之分子標誌輔助育種－目前進展與未來展望 Dr. Michael Thomson (International Rice Research Institute, Philippines)
16:15-17:00	Current Status of Breeding for Brown Planthopper resistance in Taiwan 台灣水稻抗褐飛蝨育種現況 Dr. Min-Tze Wu (吳明哲博士) (Biotechnology Division, Taiwan Agricultural Research Institute, Taiwan)

7 Oct., 2009 (Wed.)

08:00-08:30	Registration

Session 4. Utilization of Genomic Research in Rice Breeding (I)

Moderator: Dr. Yue-Ie Hsing (邢禹依教授)
Institute of Plant and Microbial Biology, Academia Sinica, Taiwan

08:30-09:15	Integration of Genomics into Breeding in Rice 基因體學研究在水稻育種上之利用 Dr. Masahiro Yano (QTL Genomics Research Center, National Institute of Agrobiological Sciences, Japan)
09:15-10:00	Production of C4 Rice－Rationale and Potential C4 水稻之生產－理論根據與可行性評估 Dr. Maurice S. B. Ku (古森本教授) (Department of Bioagriculture, National Chiayi University, Taiwan)
10:00-10:30	Tea/Coffee Break

Session 5. Utilization of Genomic Research in Rice Breeding (II)

	Moderator: Dr. Junne-Jih Chen (陳駿季副所長) **Deputy Director General, Taiwan Agricultural Research Institute, Taiwan**
10:30-11:15	Development of Single Nucleotide Polymorphism (SNP) Marker and Its Application to Breeding in Rice. SNP 分子標誌探索與水稻育種上之利用 Dr. Zixuan Wang (王子軒博士) (Plant Genome Center, Japan)
11:15-12:00	Update for Elite Rice Variety 水稻優良品種之更新 Dr. Shaoyang Lin (林少楊博士) (Honda Research Institute Japan Co. Ltd., Japan)
12:00-12:15	**Closing Ceremony** Dr. Dah-Jiang Liu, Director General of TARI 行政院農業委員會 農業試驗所 劉所長大江
12:15-13:30	Lunch
13:30-17:00	Visiting Taiwan Agricultural Research Institute

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